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19. ABSTRACT (Continue on reverse if necessary and identify by block number) Polybenzoxazoles are thermally stable, high modulus polymers made by the condensation of carboxylic acids with o-aminophenols. This report summarizes research to develop methods for the biosynthesis of substituted o-aminophenols from less expensive precursors such as substituted anilines, benzidine, and o-nitrophenols. One approach has been to exploit isolated enzymes to catalyze the desired reactions. Of the four enzymes we have studied for this purpose, tyrosinase shows the most promise. This enzyme has the desired catalytic activity, catalyzing the ortho hydroxylation of several anilines and benzidines, but the reaction rate is 100-fold slower than with phenols and an exogenous reductant must be added to the reaction to inhibit the enzyme-catalyzed oxidation of the desired o-aminophenol. As an alternative to this approach, we investigated the biosynthesis of o-aminophenols via bacterial reduction of o-nitrophenols. Bacterial strain PI1, a GE isolate, catalyzes the reduction of nitroaromatic compounds. This bacterium, identified as a Group D Streptococci, has broad substrate specificity for this nitro-reductase activity and good viability under high product and substrate concentrations. The bioreduction of nitroaromatic compoundsholds promise as a viable and attractive alternative to the chemical reduction.

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1. SUMMARY

Polybenzoxazoles are thermally stable, high-modulus polymers made by the condensation of carboxylic acids with o-aminophenols. A major obstacle to the commercialization of polybenzoxazoles is the prohibitive expense of synthesizing the o-aminophenol monomers. The chemical synthesis of o-aminophenols is accomplished by ortho nitration of the appropriate phenol followed by reduction of the o-nitrophenol. We examined the potential to biologically synthesize the o-aminophenol monomers by evaluating two approaches: use of isolated enzymes to hydroxylate aminoaromatic compounds and use of bacteria to reduce the o-nitrophenol intermediate in the chemical synthesis of o-aminophenol monomers.

Tyrosinase, p-hydroxybenzoate hydroxylase, salicylate hydroxylase, and p-aminobenzoate hydroxylase are the four classes of enzymes that were evaluated. We demonstrated that although all of these enzymes have the ability to catalyze the synthesis of an o-aminophenol, only tyrosinase shows promise as a catalyst for a general process to biosynthesize substituted o-aminophenol monomers. Tyrosinase can catalyze the o-hydroxylation of many aminoaromatic compounds, and it has the further advantage of being relatively abundant, hardy, and capable of catalyzing the desired reaction without the addition of expensive cofactors.

An alternative approach, the bioreduction of o-nitrophenols, shows promise as an effective means of synthesizing o-aminophenol monomers from the o-nitrophenol intermediate formed in the chemical synthesis of these compounds. Often, the chemical reduction of o-nitrophenols is not economically viable at the large scale due to catalyst poisoning, oxidative instability of the o-aminophenol generated in the reaction, and less than optimal yields requiring extensive product purification. Bacterial strain PI1, a GE isolate, catalyzes the reduction of nitroaromatic compounds, has broad substrate specificity for this nitro-reductase activity, and has good viability under high substrate and product concentrations. The results of our study with PI1 justify further evaluation of this route to the synthesis of o-aminophenol monomers, and may have applicability to the reduction of nitroaromatic compounds in general.

2. JUSTIFICATION

2.A. Importance of Polybenzoxazoles

Polybenzoxazoles are synthesized via the self-condensation of an o-aminophenol benzoic acid derivative (AB monomer) or by the condensation of a bis-o-aminophenol with a bis-benzoic acid derivative (AA and BB monomers) as shown in Figure 1. Polybenzoxazoles consist of a backbone of linked aromatic units and have excellent heat, strength, and solvent resistance properties. A limitation to the usefulness of polybenzoxazoles has been the inability to process these polymers by conventional methods like casting, extruding, or spinning due to the fact that they are soluble only in strong acids and have such high T_gs. This is overcome to some degree by the development of PBZ technology in which polybenzoxazoles are synthesized in polyphosphoric acid containing P_2O_5 [1,2,3]. By this method, the polymerizing mixtures can become more concentrated and form the liquid crystalline phase.

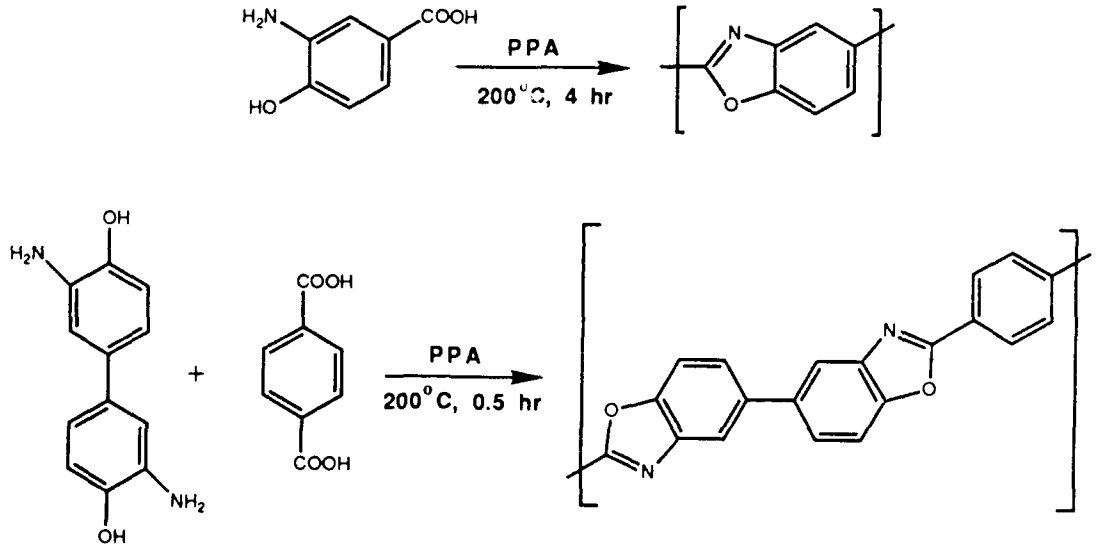


Figure 1. Synthesis of polybenzoxazoles.

Polybenzoxazoles can be classified into four groups based on differences in the degree of chain extension in solution (Figure 2) [4]. Class I polybenzoxazoles are rigid-rod homopolymers composed exclusively of rigid segments with catenation angles of nearly 180°. The high degree of chain extension in a solution of Class I polybenzoxazoles results in a low critical concentration (approximately 5%) for forming the liquid crystalline or nematic phase. Class II polybenzoxazoles are mesogenic homopolymers, composed of rigid units with catenation angles of 150°-165°. These polymers can exist in a trans or cis conformation (a "trans" conformation refers to one in which the heteroatoms in each pair of adjacent repeat units have a trans relationship; when the relationship of heteroatoms is cis, the conformation is "cis"). The trans conformation results in a chain extended form that can form the liquid crystalline phase at concentrations of approxymately 13%. Class III polybenzoxazoles are mesogenic copolymers, which incorporate rigid-rod segments in the backbone and can be liquid crystalline at solution concentrations of approximately 15%. Class IV polybenzoxazoles are nonmesogenic homopolymers which have flexible linkages between rigid backbone segments or with rigid units with catenation angles that are either below 150° or situated so that alternative sets of catenating bonds cannot be coparallel. These polymers have so many possible chain conformations that attaining the nematic phase has not been possible with the current technology.

2.B. Synthesis of Polybenzoxazole Monomers

A major obstacle preventing commercialization of polybenzoxazoles is the prohibitive expense of synthesizing the o-aminophenol monomers. The standard chemical synthesis of the o-aminophenol monomers is accomplished in two steps: first, the appropriate phenolic compound is nitrated to the corresponding o-nitrophenol, and second, the o-nitrophenol is reduced resulting in the desired monomer. Both steps of the synthesis can suffer from complications resulting in unacceptable product purity and yield.

The first step, chemical nitration, proceeds without severe complications provided that the ortho positions of the phenol are equivalent and that the para position is substituted.

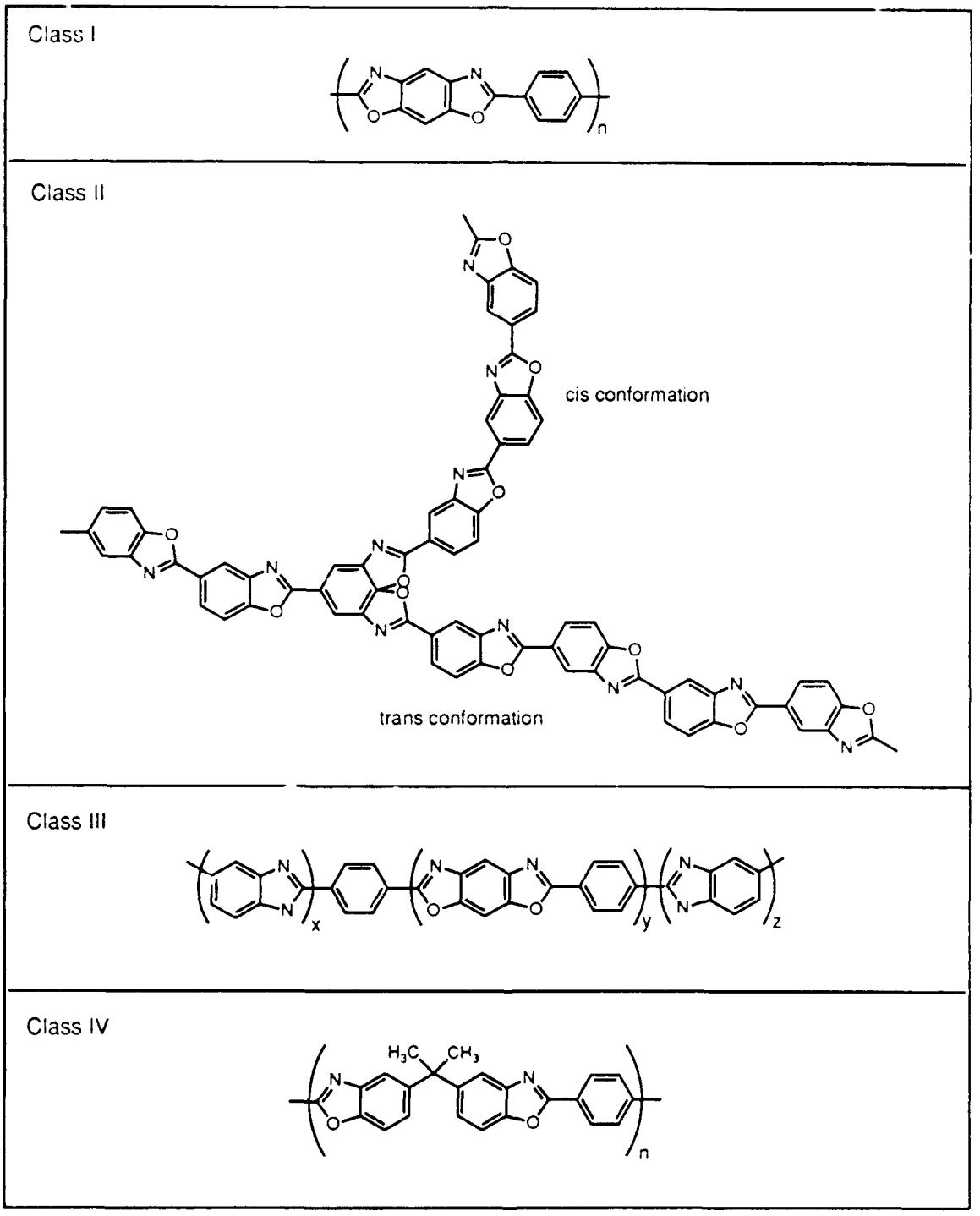


Figure 2. Four classes of polybenzoxazoles.

For example, the nitration of 4-hydroxybenzoic acid results in 87% yield of 3-nitro-4-hydroxybenzoic acid [5]. This is in contrast to the analogous nitration of 3 hydroxybenzoic acid, which results in only 18% yield of the desired 4-nitro-3-hydroxybenzoic acid and requires extensive separation of the desired product from other nitration products such as 2-nitro-3-hydroxybenzoic acid and 2,4-dinitro-3-hydroxybenzoic acid.

The second step of the synthesis of o-aminophenol monomers, reduction of o-nitrophenols, can also be plagued by complications. Reducing agents that have been used to reduce o-nitrophenols include Zn, Sn, or Fe and acid, catalytic hydrogenation, and sulfides (Figure 3). Due to side reactions and partial reactions, yields of o-aminophenols can be unacceptably low. For example, addition of mossy tin to a solution of 4-nitro-3-hydroxybenzoic acid in concentrated hydrochloric acid yields 53% 4-amino-3-hydroxybenzoic acid [5]. Under similar conditions, 3-nitro-4-hydroxybenzoic acid is reduced to 3-amino-4-hydroxybenzoic acid in 78% yield [5]. Reduction of 3,3'-dinitrobiphenol-4,4'-diol by sodium hydrosulfite results in 57% yield of 3,3'-diaminobiphenyl-4,4'-diol [6]. Catalytic hydrogenation of methyl 3-nitro-4-hydroxybenzoic acid results in 76% yield of methyl 3-amino-4-hydroxybenzoic acid [7], and the catalysts in these hydrogenations can be poisoned by the o-aminophenol products.

Yields for the reduction of o-nitrophenols are not always low; the catalytic hydrogenation of 2,2'-bis(3-nitro-4-hydroxyphenyl)propane gives very high yields (>90%) of the 2,2'-bis(3-amino-4-hydroxyphenyl)propane [8]. However, in this case, the only solvent for the hydrogenation reaction in which both the reactant and product are sufficiently soluble was dimethylformamide [8]. This solvent is not used for large scale manufacturing due to its expense and negative environmental impact. Ideally, the reduction of o-nitrophenols would be done in water to avoid the inherent environmental problems of a process using an organic solvent.

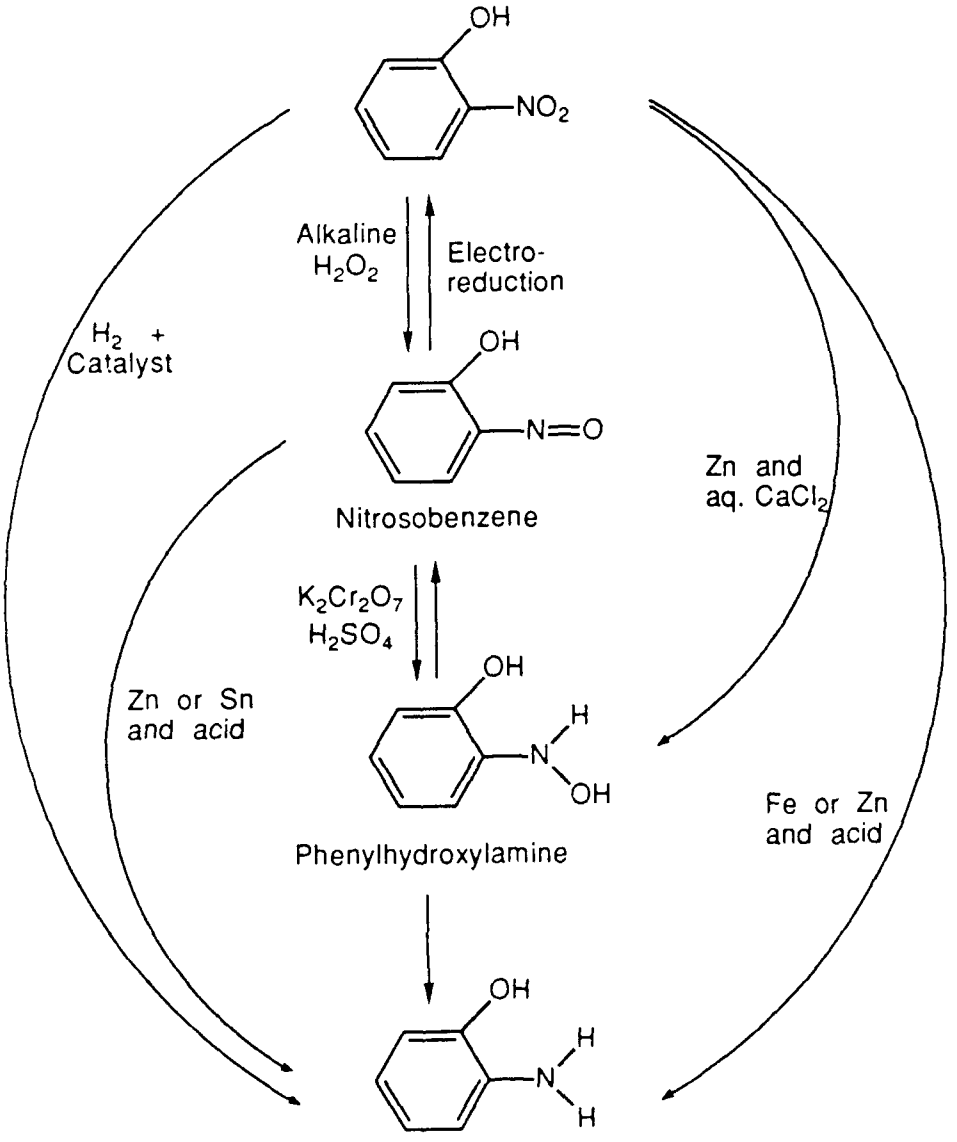


Figure 3. Chemical reduction of nitrophenols.

Low yields, presence of impurities from side reactions, catalyst poisoning in the chemical reduction of o-nitrophenols, and requirements for organic solvents are all limitations to the economic viability of the chemical syntheses of o-aminophenol monomers. Because of these difficulties, the development of a more efficient route to o-aminophenol monomers is an attractive and worthwhile goal. Biological synthesis of these compounds holds the potential of being a cost effective route to these o-aminophenol monomers. Biosynthesis of substituted o-aminophenols can be achieved by several routes: (A) hydroxylation of substituted anilines catalyzed by isolated enzymes, (B) hydroxylation of substituted anilines or acetanilides catalyzed by enzymes contained in fungus, (C) biological reduction of o-nitrophenols, and (D) production of 3-hydroxy-4-aminobenzoic acid via enzymes of the shikimic acid pathway. In this report, work to explore the feasibility of routes (A) and (C) is described.

3. APPROACH

Enzymes catalyze a variety of reactions including hydroxylations of aromatic compounds and reduction of nitroaromatic compounds. These enzymes, either as isolated proteins or in whole organisms, can be exploited to biologically synthesize substituted o-aminophenols. Biosynthesis of these o-aminophenol monomers can be achieved by several routes: (A) hydroxylation of substituted anilines catalyzed by isolated enzymes, (B) hydroxylation of substituted anilines or acetanilides catalyzed by enzymes contained in fungus, (C) biological reduction of o-nitrophenols catalyzed by bacteria, and (D) production of 3-hydroxy-4-aminobenzoic acid via enzymes of the shikimic acid pathway.

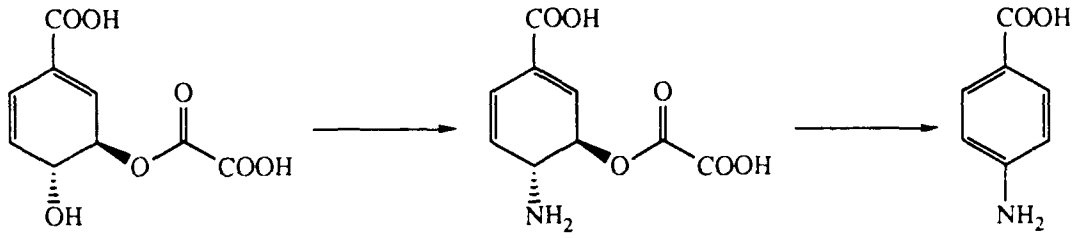
The first of these routes, the o-hydroxylation of anilines, examines the use of isolated oxidative enzymes as catlysts for this reaction. Isolated enzymes may have advantages over use of whole organisms as a catalyst in a process, especially when the whole organism is catalyzing the synthesis of a target compound via biochemistry that is not well understood. Potential advantages of a process using isolated enzymes include use of immobilization techniques which often enhance activity and stability, use of non-aqeous solvents if neccessary, and ease of product recovery. Our efforts are focused on studying o-hydroxylations of anilines catalyzed by four enzymes: tyrosinase, p-hydroxybenzoate hydroxylase, p-aminobenzoate hydroxylase, and salicylate hydroxylase. These enzymes were chosen because they hydroxylated aromatic rings in a manner that suggested that anilines would be good substrate analogs and also because these enzymes were well characterized and readily available.

The second of these routes, fungal hydroxylation of substituted anilines or acetanilides, is catalyzed by a variety of fungi [9,10,11]. For example, *Streptomyces eurocidicus* and *Streptomyces griseolus* produce both 2'- and 4'-hydroxyacetanilide in nearly equal amounts when cultured in the presence of acetanilide, while *Amanita muscaria*, *Cunninghamella blakesleeana*, *Aspergillus ochraceous*, and *Cunninghamella bainieri* produce almost exclusively the 2'-hydroxyacetanilide under similar conditions [9,10,11]. The

hydroxylation of acetanilide catalyzed by fungi is thought to occur via an arene oxide produced by the action of a cytochrome P450-like monooxygenase, and the substrate specificity of these fungal monooxygenases is broad. One advantage of this approach is the enhanced oxidative stability of the product; however, the expression of proteins in fungi is often developmentally regulated and so an efficient process using fungi as the catalyst may require intensive research effort to develop.

The third route, biological reduction of o-nitrophenols, is an alternative to chemical reduction of the o-nitrophenol intermediates in the synthesis of o-aminophenol monomers. Reduction of nitroaromatic compounds to the corresponding aminoaromatic compounds is catalyzed by a variety of microorganisms [12,13,14,15]. The reduction occurs under anaerobic conditions and can be catalyzed by whole organisms. Advantages of this route to o-aminophenols include production of the oxidatively unstable product under oxygen-free conditions, tolerance of the bacteria to relatively high concentrations of substrate and product, and presumably simple product recovery process due to the neat conversion of the o-nitrophenol to the o-aminophenol.

The fourth route to the biosynthesis of o-aminophenol monomers makes use of the enzymes in the shikimic acid pathway to produce 3-amino-4-hydroxybenzoic acid. 4-Amino-4-deoxychorismic acid, derived from the chorismic acid intermediate in the shikimic acid pathway, is transformed to 4-aminobenzoic acid by 4-aminobenzoate synthase (Figure 4). Mutants lacking 4-aminobenzoate synthase accumulate 4-amino-4-deoxychorismic acid in the supernatant, and this can then be chemically converted to 4-amino-3-hydroxybenzoic acid [16]. A major advantage of this route is that the 4-amino-deoxychorismic acid can be obtained by fermentation of the appropriate bacterium with an inexpensive carbon source. A major disadvantage is that this approach leads to the synthesis of a single compound; there is little evidence that this process could be generalized to produce a variety of substituted o-aminophenols.



Chorismic Acid

4-Amino-4-deoxy-chorismic Acid

4-Aminobenzoic Acid

Figure 4. Transformations of chorismic acid.

While all of these routes are possible approaches to the biosynthesis of o-aminophenol monomers, we have focused our efforts on route (A), use of isolated enzymes to catalyzed the hydroxylation of substituted anilines, and route (C), use of bacteria to catalyze the reduction of o-nitrophenols. Route (A) was chosen because a literature survey of enzymes revealed that several had the potential to catalyze the desired hydroxylation reactions and use of isolated enzymes has the advantages outlined in the discussion above. Route (C) was chosen because this reduction proved to be straightforward with high yields and high conversions, and therefore offers an attractive alternative to chemical reduction.

4. RESULTS

4.A. Tyrosinase

4.A.1. Introduction

Tyrosinase catalyzes the conversion of phenols to o-quinones in the presence of oxygen (Figure 5) [17]. This reaction occurs in two steps: (1) ortho-hydroxylation of phenol with oxygen ("hydroxylase" activity), and (2) subsequent oxidation of the catechol to the o-quinone ("catecholase" activity). The monooxygenase activity of tyrosinase is unusual in not requiring a classical coenzyme. Instead, two copper atoms are bound per active site, and this binuclear copper center provides the binding site for the substrates (phenol and oxygen) as well as the electrons needed in the reaction [18]. The catalytic mechanism for tyrosinase (shown in Figure 6) is thought to involve binding of the phenolic oxygen atom and molecular oxygen to the copper atoms in the active site. This is followed by hydroxylation of the phenol at the nearby ortho position. The catechol is then oxidized by the enzyme to the o-quinone, thereby reducing the copper atoms in the active site and generating the catalytic form of the enzyme ready for another cycle.

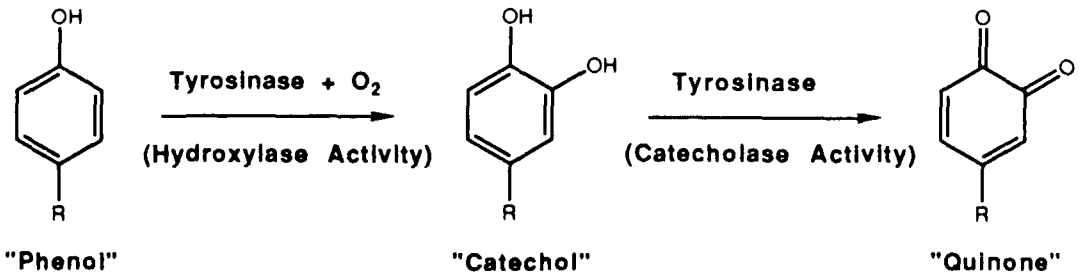


Figure 5. Two-step reaction of tyrosinase with phenols.

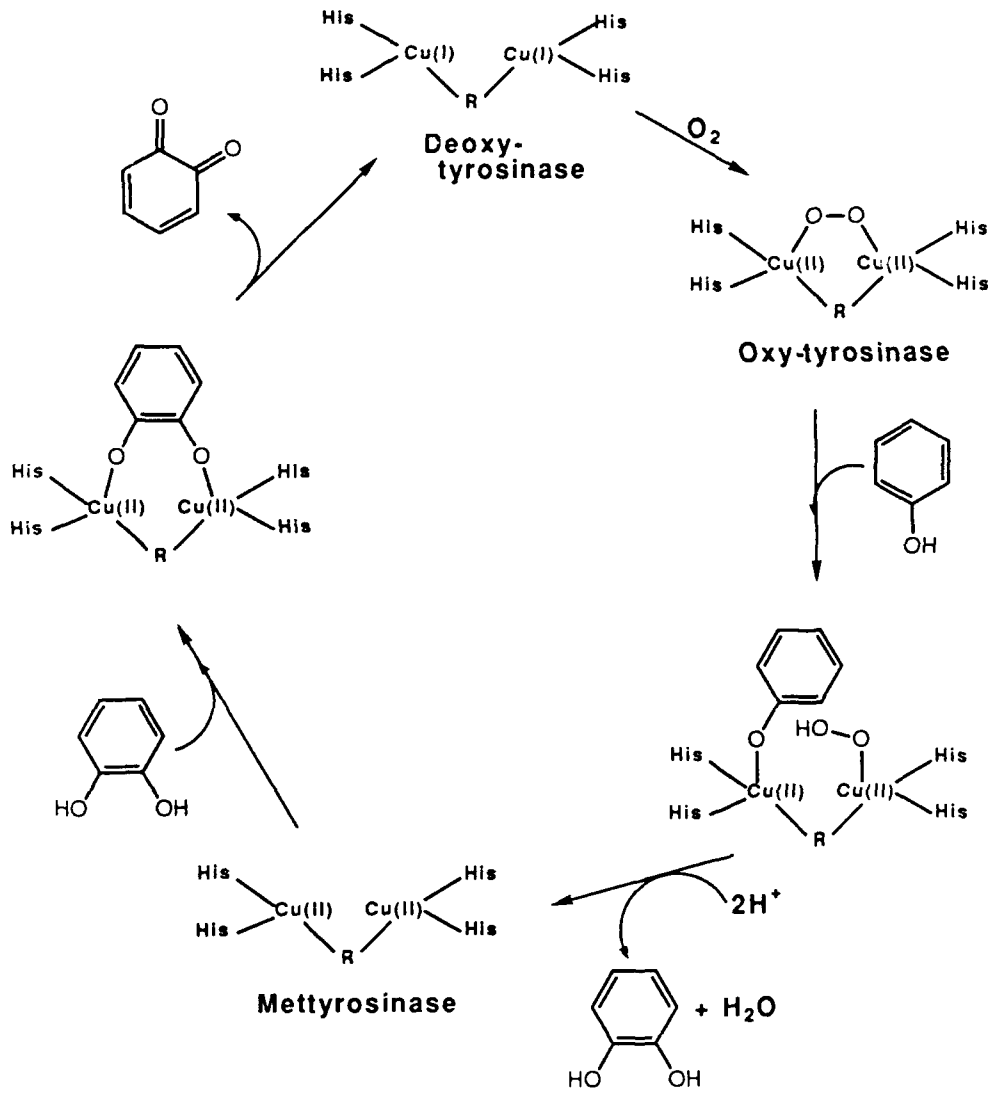


Figure 6. Proposed mechanism for the reaction of phenol with tyrosinase.

Since the copper at the active site should bind nitrogen as well as oxygen, compounds such as aniline and substituted anilines may be ortho hydroxylated by tyrosinase. There is chemical precedence for the oxidation of aromatic amines by copper-amine complexes [19]. However, the product of the chemical hydroxylation of aniline by copper complexes is not the desired o-aminophenol, but instead, is a mixture of products including 2-amino-5-anilino-quinone-4-anil. This generally arises from coupling between unreacted aniline and the

oxidized form of the hydroxyaniline. By using tyrosinase, the substrate can be oriented to produce exclusively ortho-hydroxylation, and the subsequent oxidation to the reactive imino-quinone may be inhibited by the addition of the appropriate reductant.

Tyrosinase is widely distributed in both the plant and animal kingdoms at all phylogenetic levels, with enzyme specificity generally becoming narrower at the higher end of the phylogenetic scale [20,21]. Multiple forms of the enzyme can exist in the same organism. For instance, there are four isoforms of tyrosinase in mushrooms [22], two isoforms in the eubacterium *Vibrio tyrosinaticus* [23], four isoforms in the frog *Rana pipiens* [24], three isoforms in potatoes [25], and only one form of tyrosinase in *Neurospora crassa* [26]. The ratio of hydroxylase to catecholase activity can vary from one source of tyrosinase to another, and even within isoforms of tyrosinase found in the same organism. For example, the α, β, γ, and δ isoforms of mushroom tyrosinase have ratios of catecholase to hydroxylase activities of 2.4, 5.25, 12.4, and 14.3, respectively [27].

Tyrosinase has a very broad substrate specificity. Substrates for the hydroxylase activity include many substituted phenols and, as we have demonstrated concurrently with Toussaint and Lerch [28], several substituted anilines. Substrates for the catecholase activity include a wide range of o-diphenols but not m- or p-diphenols [18]. Generally, K_m and V_{max} values are lower for substrates with electron withdrawing substituents on the aromatic ring. A general feature with catecholase substrates, especially catechol, is the nonlinear curve of the progress of the reaction presumably due to inactivation of the enzyme by the o-quinone [18].

Tyrosinase is an attractive enzyme catalyst for the synthesis of substituted o-aminophenol monomers because it is relatively abundant, requires no expensive cofactors for activity, is active in some hydrophobic solvents, can hydrolyze a number of substrates, and holds the possibility of immobilization in a bioreactor. The major disadvantage that must be overcome is the tyrosinase-catalyzed oxidation of the desired o-aminophenol product.

4.A.2. Pre-contract Effort

We determined that a commercially available preparation of mushroom tyrosinase catalyzes the regiospecific hydroxyaltion of aniline, substituted anilines, and benzidine derivatives to produce o-aminophenols. However, these o-aminophenols are further oxidized by the enzyme to produce iminoquinones, which are susceptible to nucleophilic attack by unreacted anilines. With aniline as the substrate, this results in the formation of 2-amino-5-anilino-4-anil, as shown in Figure 7. When 2-aminophenol is combined with the enzyme, the formation of the iminoquinone is deduced from the structure of the isolated product, 3-aminophenoxaz(2)one (shown in Figure 7). Investigations of the reaction of aniline and tyrosinase in nearly anhydrous organic solvents also yielded the phenoxazone.

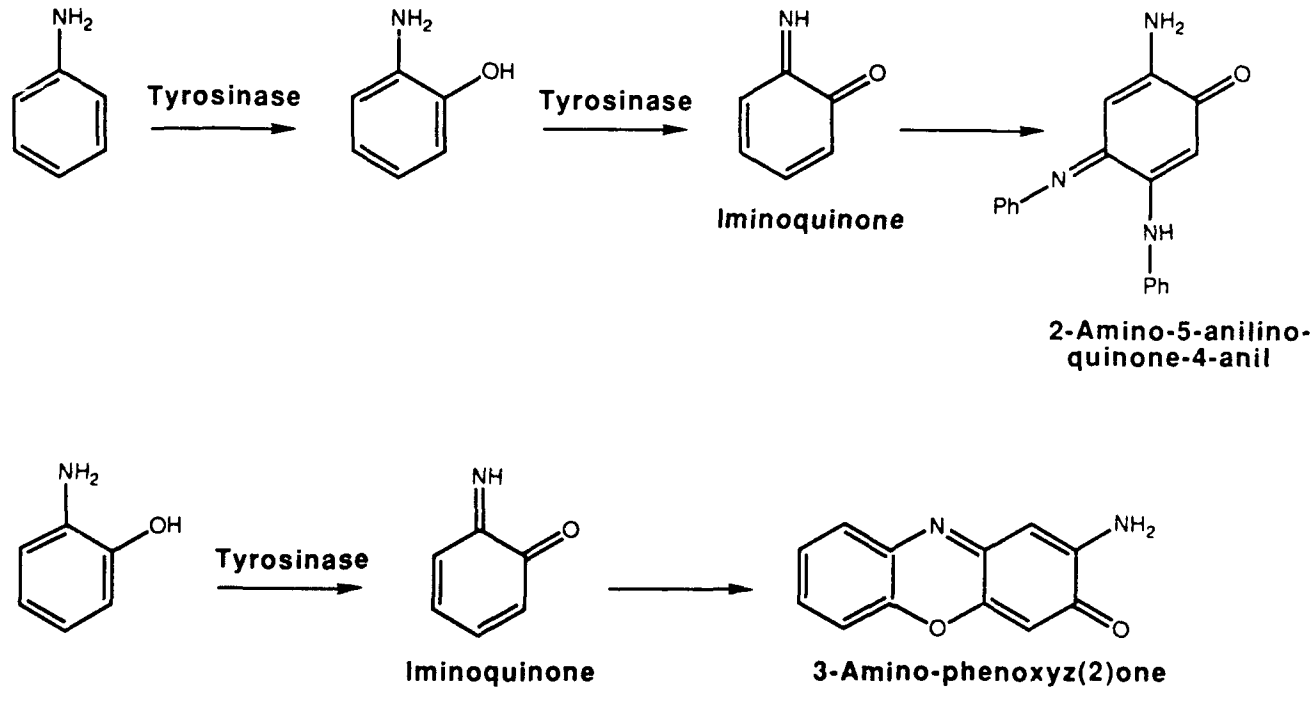


Figure 7. Reaction of tyrosinase with aniline and 2-aminophenol. Reactions are done in aqueous 50 mM phosphate buffer, pH 7.4, containing 0.1 mg tyrosinase/ml and 2 mM substrate.

4.A.3. Results of Contract Effort.

Mushroom Tyrosinase: Initially, experiments were done with mushroom tyrosinase, a source of tyrosinase that is well-studied and commercially available from Sigma as a partially purified enzyme preparation. Assays typically were done at 30°C in 50 mM phosphate buffer, pH 7.4, containing 2 mM substrate (added from a 1 M stock in acetonitrile), 1.1 mM dissolved oxygen, and 0.1 mg/ml tyrosinase. These assay conditions were the result of preliminary experiments that indicated that the rate of conversion of aniline to products catalyzed by mushroom tyrosinase is relatively constant over the pH range of 6 to 9. In addition, exposure of mushroom tyrosinase to organic solvents does not lead to severe inhibition of enzyme activity as evidenced by the following experiments: (1) assays of tyrosinase with o-cresol in either water, 15% acetonitrile, or 25% methanol over several hours result in no detectable decrease in the rate of o-cresol oxidation, and (2) tyrosinase catalyzes the oxidation of o-cresol even when the assays are done in water-saturated water-immiscible organic solvents like diethyl ether, dimethyl carbonate, and chloroform.

Progress of the tyrosinase reactions is measured by two methods: (1) oxygen consumption is monitored with a YSI oxygen electrode at 30°C, or (2) substrate depletion and/or product formation are quantified by HPLC analysis of these compounds from an assay. The HPLC analysis consists of loading the assay mixture onto a C_{18} column and eluting the compounds of interest in a linear gradient from 20 mM Tris, pH 8.0, to acetonitrile; detection is at 254 nm.

The tyrosinase-catalyzed hydroxylation of aniline is used as a model system to study the ability of mushroom tyrosinase to hydroxylate substituted anilines and thereby produce useful o-aminophenol monomers for polybenzoxazole synthesis. The product isolated from the reaction of tyrosinase with aniline in a typical assay is not the desired o-aminophenol, but rather is the 2-amino-5-anilino-4-anil (see Figure 7). This is most likely attributed to the enzyme-catalyzed oxidation of o-aminophenol to the imminoquinone, which can react with aniline to form the 2-amino-5-anilino-4-anil. The effect of aniline concentration on enzyme

activity was also examined, and the results are shown in Figure 8. Mushroom tyrosinase is quite sensitive to aniline concentration and has only half of the maximal rate when 50 mM aniline was present. This is in contrast to the oxidation of o-aminophenol catalyzed by tyrosinase, in which the rate of o-aminophenol oxidation was unaffected by concentrations of o-aminophenol as high as 200 mM. Thus, the apparent inhibition of tyrosinase in the presence of high concentrations of aniline is not due to o-aminophenol inhibition.

The formation of the highly reactive iminoquinone in the reaction between aniline and tyrosinase is a problem that had to be addressed for this to be a fruitful route to the synthesis of o-aminophenols. The addition of ascorbic acid to the reaction of tyrosinase and tyrosine prevents the further oxidation of the catechol product and permits quantitative conversion of tyrosine to dopa [29]. Therefore, the use of reductants in the tyrosinase reaction with aniline was studied as we searched for a means of stabilizing the desired o-aminophenol product and reducing the enzyme back to its initial oxidation state. Figure 9 summarizes the data from an experiment to determine the effect of three different reducing agents on tyrosinase activity with two substrates, p-cresol and aniline. With p-cresol as the substrate, the addition of ascorbic acid to the assay actually increases the reaction rate, presumably by reducing the Cu(II) to Cu(I) and thereby enhancing the rate of the catalytic cycle. Other reductants (dithiothreitol and dithionite) inhibit enzyme activity. When aniline is the substrate, even ascorbic acid inhibits the tyrosinase-catalyzed hydroxylation of aniline, as determined by HPLC analysis and quantitation of aniline and o-aminophenol in the assays. Therefore we conclude that addition of ascorbic acid to a reaction between mushroom tyrosinase and aniline does not provide a means for stabilizing the desired o-aminophenol product.

Another approach to stabilization of the desired o-aminophenol products is to study the hydroxylation of substrate analogs that, when hydroxylated, have enhanced stability towards further oxidation. Thus, the hydroxylase activity of mushroom tyrosinase with acetanilide was studied. If hydroxylated, the product from this substrate would have enhanced oxidative stability over the product from the non-acetylated aniline analogs.

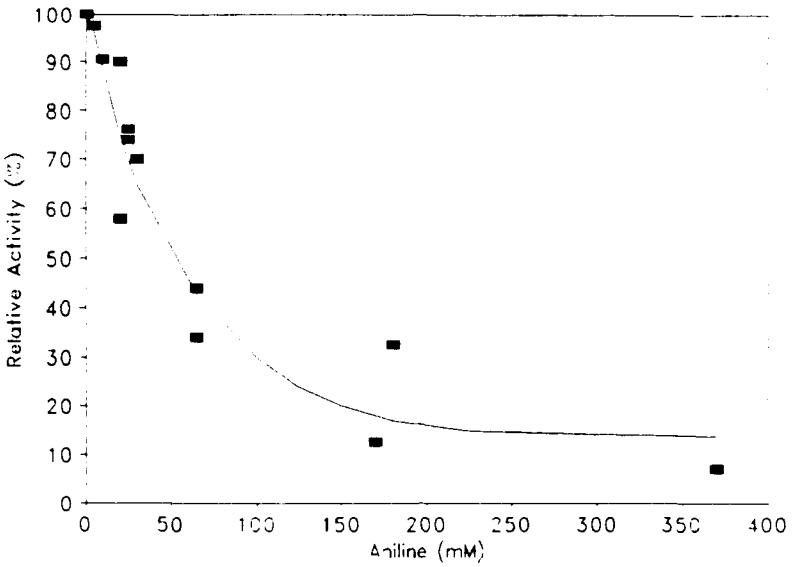


Figure 8. Effect of aniline concentration on the rate of oxygen consumption in tyrosinase assays.

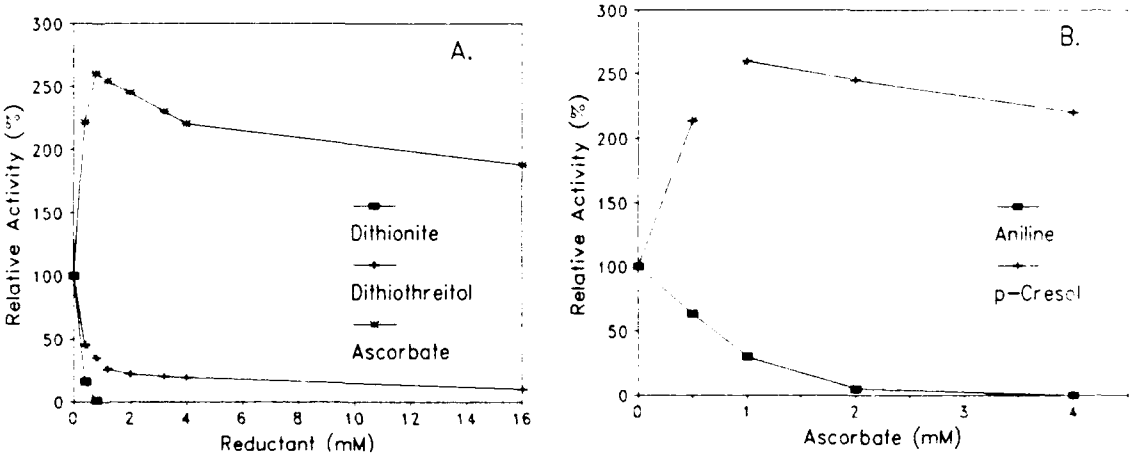


Figure 9. Effect of reductants on tyrosinase activity. Reaction rates were determined from the rate of oxygen consumption. Assay of tyrosinase with p-cresol and reductant (A), and effect of ascorbic acid concentration on the reaction of tyrosinase with p-cresol or aniline (B).

For example, incubation of 2′-hydroxyacetanilide with mushroom tyrosinase does not result in the oxidation of the 2′-hydroxyacetanilide, indicating that this compound is stable in the presence of the enzyme and cannot reduce the copper in the active site of met-tyrosinase back to deoxy-tyrosinase. Assays of tyrosinase with acetanilide were carried out with either a reductant to convert the met-tyrosinase to deoxy-tyrosinase or H_2O_2 to directly convert met-tyrosinase to oxy-tyrosinase. The results with mushroom tyrosinase were ambiguous; we observed loss of acetanilide in the assays as determined by HPLC analysis of the reaction mixture, but we could not isolate any hydroxylated product.

Although the hydroxylation of aniline is a useful model reaction for studying tyrosinase, the resulting o-aminophenol is not a monomer for polybenzoxazole production. Therefore, the substrate specificity of mushroom tyrosinase was examined. This was done by monitoring oxygen concentration in enzyme assays with several substrate analogs. Since the mushroom tyrosinase preparation is impure, the results (summarized in Table 1) can be used to ascertain relative enzyme activities and not maximum specific activities. Note that the oxygen is consumed at a much faster rate in the reaction of o-aminophenol as compared to the reaction with aniline. This implies that the rate of hydroxylation of aniline to o-aminophenol may be much slower than the subsequent oxidation, and hydroxylation may be the rate limiting step in this catalytic sequence. Also note that the addition of a 4-methyl group to aniline or phenol significantly enhances the rate of oxygen uptake. This implies that mushroom tyrosinase may have a preference for 4-substituted substrates (the natural substrate, tyrosine, is a 4-substituted phenol). Other aromatic amines that cause slow but significant oxygen uptake when incubated with mushroom tyrosinase were 2,4-dimethylaniline, 4-methylaniline, 3,4-dimethylaniline, 4-aminoaniline, 4-aminoacetanilide, 4-amino-phenylalanine, benzidine, and o-tolidine.

A range of amino- and hydroxy-substituted benzoic acids were also assayed with mushroom tyrosinase. These assays were done in oxygen-saturated water and water-saturated organic solvents. Aqeuous assays contain 100 mM phosphate buffer, pH 7.0, 2.5 mM substrate, and 0.2 mg/ml tyrosinase. Organic assays contain 10 mM substrate, 0.2 mg/ml

Table 1. Relative activity of tyrosinase with various substrates.

(1 U = 1μmol of oxygen consumed / minute)

Substrate	Specific Activity (U/mg)	% Relative Activity
tyrosine	188	100
o-aminophenol	115	61
p-cresol	109	54
p-hydroxy-acetanilide	39	21
phenol	36	19
p-methylaniline	11	6
aniline	6	3
others (see text)	<2	<1

tyrosinase, and either no reductant, 1 mM ascorbic acid, or 1 mM dithiothreitol (DTT) in a reaction volume of 5 ml of water-saturated solvent (diethyl ether, dimethyl carbonate, or chloroform). The substrates examined included 2-, 3-, and 4-hydroxybenzoic acid; 2-, 3-, and 4-aminobenzoic acid; the corresponding methyl esters of these same acids; and 4-aminocyanobenzene and 4-hydroxycyanobenzene. Substrates and products are separated and quantified by HPLC analysis of the assay mixtures, which are loaded onto a C_{18} column and eluted with a linear gradient from 0.1% trifluoroacetic acid in water to acetonitrile containing 0.1% trifluoroacetic acid. Detection of compounds is done spectrophotometrically at 260 nm, 280 nm, and 300 nm. No product peaks were detected and no significant changes in the peak areas of substrates were observed in both aqeous and organic reactions. These results demonstrate that both hydroxyl and amino substituted benzoic acids are not hydroxylated by mushroom tyrosinase. Thus, mushroom tyrosinase cannot be used as a catalyst to directly hydroxylate aminobenzoic acids to make AB monomers such as 4-amino-3-hydroxybenzoic acid.

Benzidine and o-tolidine are examples of other compounds that are hydroxylated by tyrosinase to produce potentially useful polybenzoxazole monomers. These compounds were known to be substrates for tyrosinase from our earlier research efforts, but the products had not been characterized. The tyrosinase assays with these substrates were done in 50 mM phosphate buffer, pH 7.4, containing 2 mM substrate, 1.1 mM dissolved oxygen, and 0.1 mg/ml mushroom tyrosinase. With o-tolidine as the substrate, one major product is isolated. Mass spectrometry of this product has a parent ion of 660 and is consistent with the structure shown in Figure 10. Ortho-tolidine is apparently hydroxylated and then further oxidized to the iminoquinone derivative which undergoes further oxidation, analogous to the reaction with aniline. With benzidine as the subatrate, again one major product is isolated. The mass spectrum of this compound has a parent ion of 380 and is consistent with hydroxylation of one ring, followed by iminoquinone formation and benzidine addition to the iminoquinine. These results demonstrate that mushroom tyrosinase can catalyze the hydroxylation of a biphenyl structure, and this hydroxylation may provide a route to the synthesis of AA monomers if the oxidation of the hydroxylated product can be prevented.

Other Tyrosinases: There are many different potential sources of tyrosinase, each unique in the ratio of hydroxylase to catecholase activities. In addition, some sources of tyrosinase produce several isoforms with varying ratios of these activities. The commercially-available, partially purified mushroom preparation of tyrosinase contains approximately 5% tyrosinase in four isoforms. The supplier (Sigma) has communicated that the enzyme preparation is highly variable in isoform composition from batch to batch due in part to the fact that mushroom growers are cultivating strains with decreased levels of tyrosinase. Because the mushroom tyrosinase has high catecholase activity relative to hydroxylase activity and is so variable from batch to batch, we decided to study the catalytic properties of the potato and *Neurospora crassa* tyrosinases. The potato enzyme is commercially available and reported to have reduced catecholase activity relative to that of mushroom tyrosinase [30]. However, we have found no evidence of hydroxylation of anilines when incubated with the potato tyrosinase under a variety of conditions.

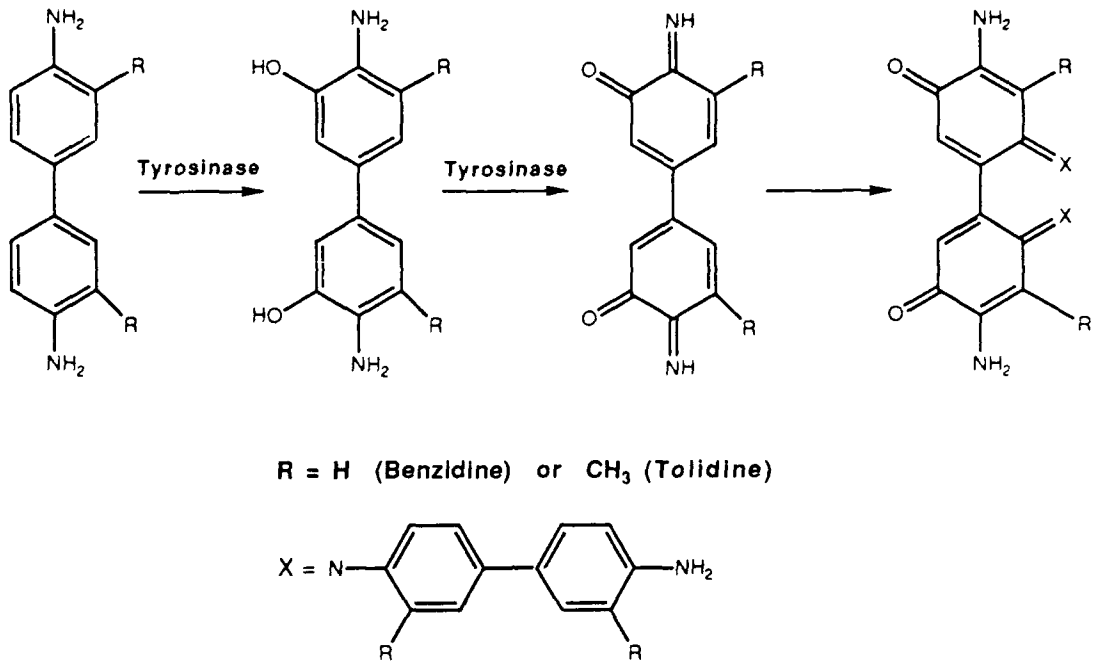


Figure 10. Proposed pathway for reaction of diaminobiphenols with tyrosinase.

Tyrosinase isolated from *Neurospora crassa* is well-characterized, exists as only one form in any particular strain, is reported to hydroxylate anilines to o-hydroxyanilines, and is not inhibited by ascorbic acid as is the mushroom tyrosinase [28,31]. Tyrosinase was purified from *Neurospora crassa* strain FGSC320-T$^{(L)}$, a strain that overproduces tyrosinase, by the procedure of Lerch [26]. A summary of the purification is shown in Table 2. As the purification progresses and the tyrosinase becomes more pure and concentrated, the specific activity becomes more unstable. In the tyrosinase fractions from the last chromatography column (hydroxylapatite) in the purification, the half-life of the tyrosinase activity is approximately 24 hours at 4°C. SDS polyacrylamide gel electrophoresis of the purified tyrosinase shows one band of approximately 46,000 molecular weight, which is consistent with data from Lerch [26]. The tyrosinase was stored in small aliquots at -20°C in 30% glycerol; under these storage conditions the enzyme activity remains stable.

Table 2. Purification of tyrosinase from *Neurospora crassa*.

Step	Volume (ml)	Total Activity (units$X10^{-3}$)	Total Protein (mg)	Specific Activity (units/mg)	% Yield
1. Dialysis after $(NH_4)_2SO_4$ precipitation	160	98.3	389	253	100
2. CM cellulose eluate	260	44.9	185	243	45.6
3. DEAE Sephadex eluate	380	12.5	75	167	12.7
4. Hydroxylapatite eluate	65	5.1	2	2550	5.2

An alternative isolation protocol for *Neurospora crassa* tyrosinase was developed. This procedure simply involves pulverizing the frozen mycelium into a powder, suspension of that powder in a phosphate buffer, pH 7.2, and then precipitaion of the tyrosinase in the crude extract with ammonium sulfate. The pellet from the 30%-60% ammonium sulfate cut was resuspended in phosphate buffer, dialyzed against phosphate buffer containing 30% glycerol, and stored at -20°C. This crude tyrosinase preparation has a specific activity of 47 units/mg protein, and can be stored for at least 3 months at -20°C with no apparent loss of activity. Because the crude preparation does not contain impurities that inhibit the desired catalytic reaction and because the stability is actually greater than the pure tyrosinase, this simplified isolation protocol is used for routine isolation of tyrosinase.

Preliminary experiments with *Neurospora crassa* tyrosinase demonstrated that this source of the enzyme does catalyze the hydroxylation of aniline and p-aminotoluene. A detailed investigation of this tyrosinase began with a study to understand the effect of pH on the reaction rate. This was accomplished by determining the rate of oxygen uptake in air-saturated solutions ranging in pH from 5 to 8 and containing *Neurospora* tyrosinase and p-cresol or p-aminotoluene. Assays with p-cresol contain 20 mM sodium phosphate, 2 mM p-cresol, and 0.6 units of tyrosinase in a total volume of 3 ml (one unit of tyrosinase is

defined as the amount of enzyme that catalyzes the oxidation of 1 μmole L-DOPA per minute at 30°C). Assays with p-aminotoluene contain 20 mM sodium phosphate, 2 mM p-aminotoluene, 4 mM ascorbic acid, and 9 units of enzyme in a total volume of 3 ml. After preincubating the air-saturated solution of buffer and substrate at 30°C, the reaction is initiated by the addition of enzyme. Oxygen uptake is recorded using the YSI oxygen monitor and probe, and the rate of oxygen uptake is calculated from the linear portion of the recorded curve. Assays are done in duplicate, and the reaction rates are reported as nmole oxygen consumed per minute per unit of tyrosinase. The data from this pH study is shown in Figure 11. With p-cresol as the substrate, there is a broad pH maximum between 6.5 and 8.0 with a sharp decrease in activity below pH 6.5; this is very similar to the pH response of the mushroom tyrosinase. With p-aminotoluene as the substrate, the reaction rates are at least 10-fold slower than with p-cresol and show an increase as the pH is increase to 7.5. The pK_a of p-aminotoluene is approximately 5, so an increase in pH of the assay will increase the proportion of non-protonated p-aminotoluene, the compound that more closely resembles the natural, non-charged substrates like p-cresol.

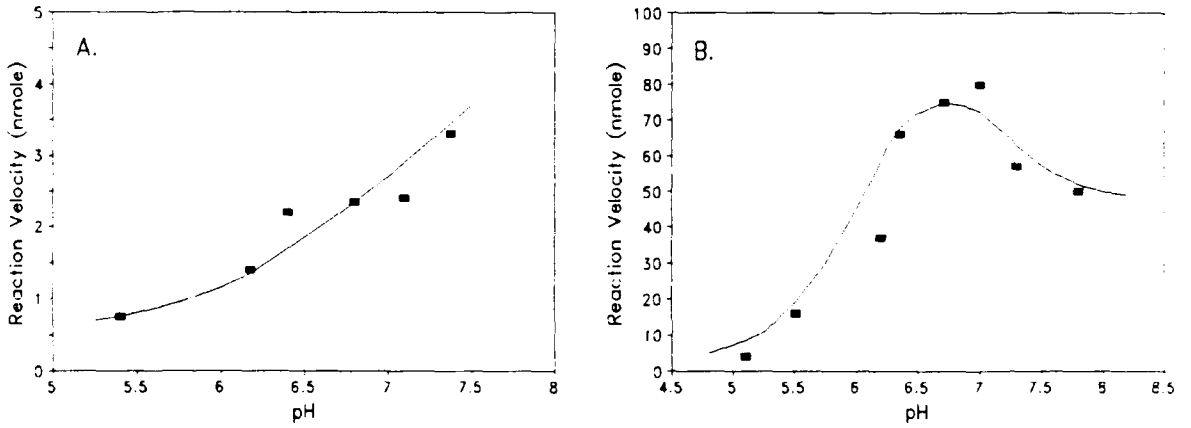


Figure 11. pH Profile for the tyrosinase reaction. Substrate is p-aminotoluene (A) or p-cresol (B). Reaction velocity expressed in nmole O_2 uptake per minute per unit of enzyme.

The substrate specificity of *Neurospora crassa* tyrosinase was studied using 4'-amino-biphenyl-4-carboxylic acid, p-aminotoluene, benzidine, and bis(4-aminophenyl)ether (Figure 12). A typical reaction contains 23 units/ml of crude *Neurospora* tyrosinase, 50 mM phosphate buffer (pH 7.2), 15 mM ascorbic acid, and 2.5 to 20 mM substrate. The assay mixture is thoroughly oxygenated prior to the addition of ascorbic acid, and the reaction is initiated by the addition of the substrate. Frequently, additional ascorbic acid is added during the reaction to maintain a significant concentration of reduced ascorbic acid in the assay. The progress of the reaction is followed by quantifying the substrates and/or products; this is accomplished by analyzing aliquots from the reaction with HPLC. The column used is a Whatman partishere C_{18} column, and the compounds of interest are eluted with a linear gradient from 50 mM hexanesulfonic acid (pH 3.5) to 80% acetonitrile in water containing 50 mM hexanesulfonic acid. The wavelength for detection of eluted compounds varies depending on the substrate being examined.

With this assay protocol, the reaction between benzidine and tyrosinase was examined. In 1.4 hours, 64% of the 2.5 mM benzidine in the reaction was hydroxylated to 3-hydroxybenzidine and 3,3'-dihydroxybenzidine (see Figure 12). The substrate and the mono- and di-hydroxylated products are well resolved by HPLC (Figure 13). After 1.4 hours, the reaction was stopped, and the substrate and products were extracted from the reaction mixture with ethyl acetate. The ethyl acetate was evaporated under nitrogen, and the residue was dissolved in Tri-Zil Z (a mixture of trimethylsilyimidazole in dry pyridine that is sold by Pierce and is useful for silanating hydroxyl compounds without derivitizing amines). After stirring overnight, the sample was analyzed by gas chromatography (GC) / mass spectroscropy (MS). The resulting data confirms the presence of mono- and di-hydroxylated products in the reaction mixture (Figure 14). One compound with a GC retention time of 18 minutes has a parent ion of 272, the correct molecular weight for derivitized 3-hydroxybenzidine. Another compound with a GC retention time of 20 minutes has a parent ion of 360, the correct molecular weight for derivitized 3,3'-dihydroxybenzidine. In other reactions between benzidine and tyrosinase, the yield of dihydroxylated product can be increased by a series of additions of tyrosinase and ascorbic acid to the reaction.

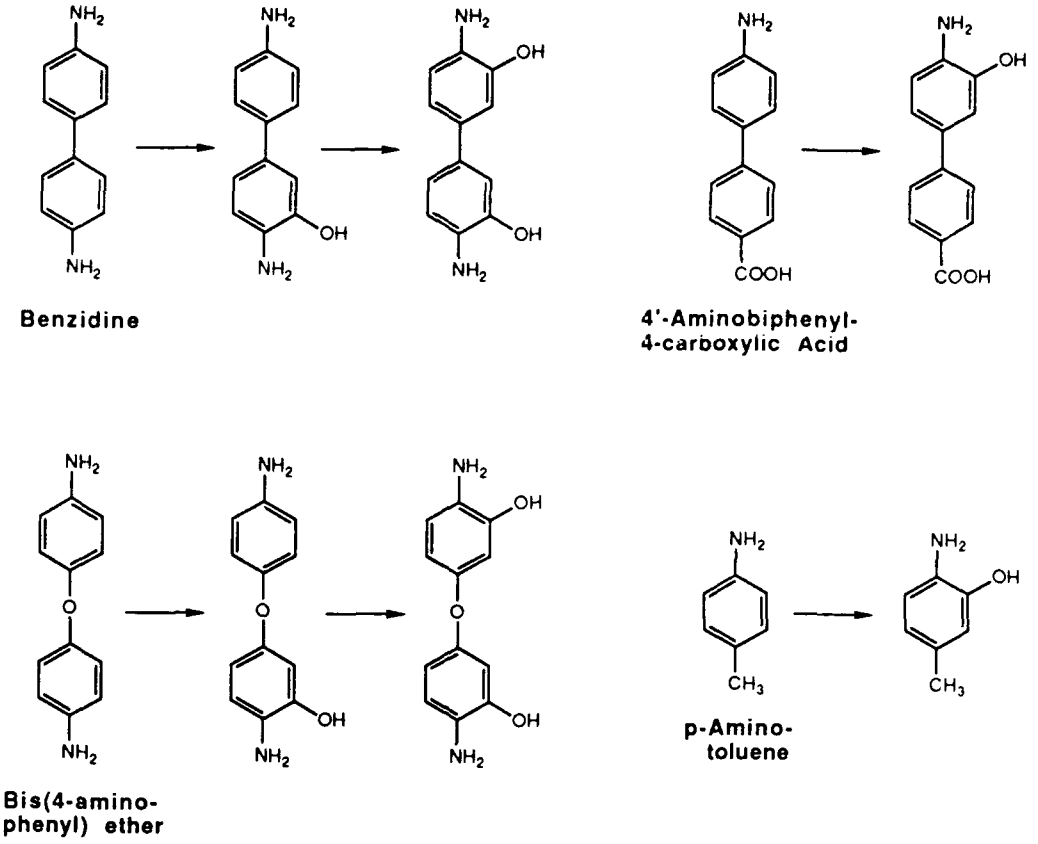


Figure 12. Substrates for ortho-hydroxylation by tyrosinase.

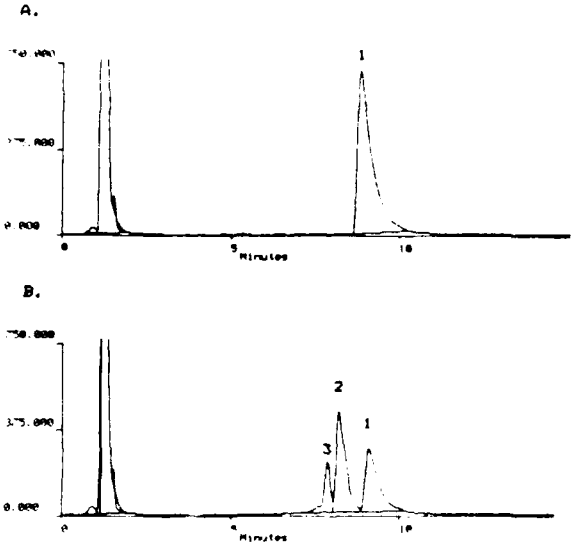


Figure 13. HPLC analysis of the tyrosinase-catalyzed hydroxylation of benzidine. Analysis of the initial time point (A) and one hour after initiating the reaction (B). Peak 1 is benzidine; peak 2 is monohydroxybenzidine; peak 3 is dihydroxybenzidine.

The difficulty in achieving 100% conversion to dihydroxylated product is maintaining the reducing conditions neccessary to prevent oxidation of this product, especially when there is high concentration of product and low concentration of substrate.

The reaction between p-aminotoluene and tyrosinase was also examined (see Figure 12). In 1.5 hours, the reaction of 20 mM p-aminotoluene and *Neurospora* tyrosinase resulted in a 27% conversion of the substrate to 4-amino-3-hydroxytoluene. At this point, the reaction was stopped, and the product and substrate were extracted with ethyl acetate. The ethyl acetate was removed under a stream of nitrogen, and BSTFA (N,O-bis(trimethyl-silyl)trifluoroacetamide, a trimethylsilyl donar used to derivitize hydroxyl and amino groups) was added to derivitize the substrate and product. The derivitized compounds were then separated and analyzed by GC/MS. The GC peaks were well separated, with the mass spectrum of one peak corresponding to derivitized substrate (molecular ion of 179) and the mass spectrum of another peak corresponding to derivitized 4-amino-3-hydroxytoluene (molecular ion of 267). In other reactions between p-toluidine and tyrosinase, the conversion of substrate to 4-amino-3-hydroxytoluene was as high as 97%.

Since bis(3-hydroxy-4-aminophenyl)ether may be an interesting monomer for polybenzoxazoles, the reaction between tyrosinase and bis(4-aminophenyl)ether was examined under the typical assay conditions described earlier. After 2 hours, 34% of the 2.5 mM bis(4-hydroxyphenyl)ether in the assay had been monohydroxylated (see Figure 12). Confirmation of this was obtained by extracting the reaction mixture with ethyl acetate, evaporating the ethyl acetate under nitrogen, derivitizing the substrate and product with BSTFA, and analyzing the sample by GC/MS. The GC peaks were well separated, with the mass spectrum of one peak corresponding to derivitized substrate (parent ion of 344) and the mass spectrum of another peak corresponding to the monohydroxylated product (parent ion of 432). Dihydroxylated product was never detected, perhaps due to the low solubility of the bis(4-aminophenyl)ether substrate and monohydroxylate product

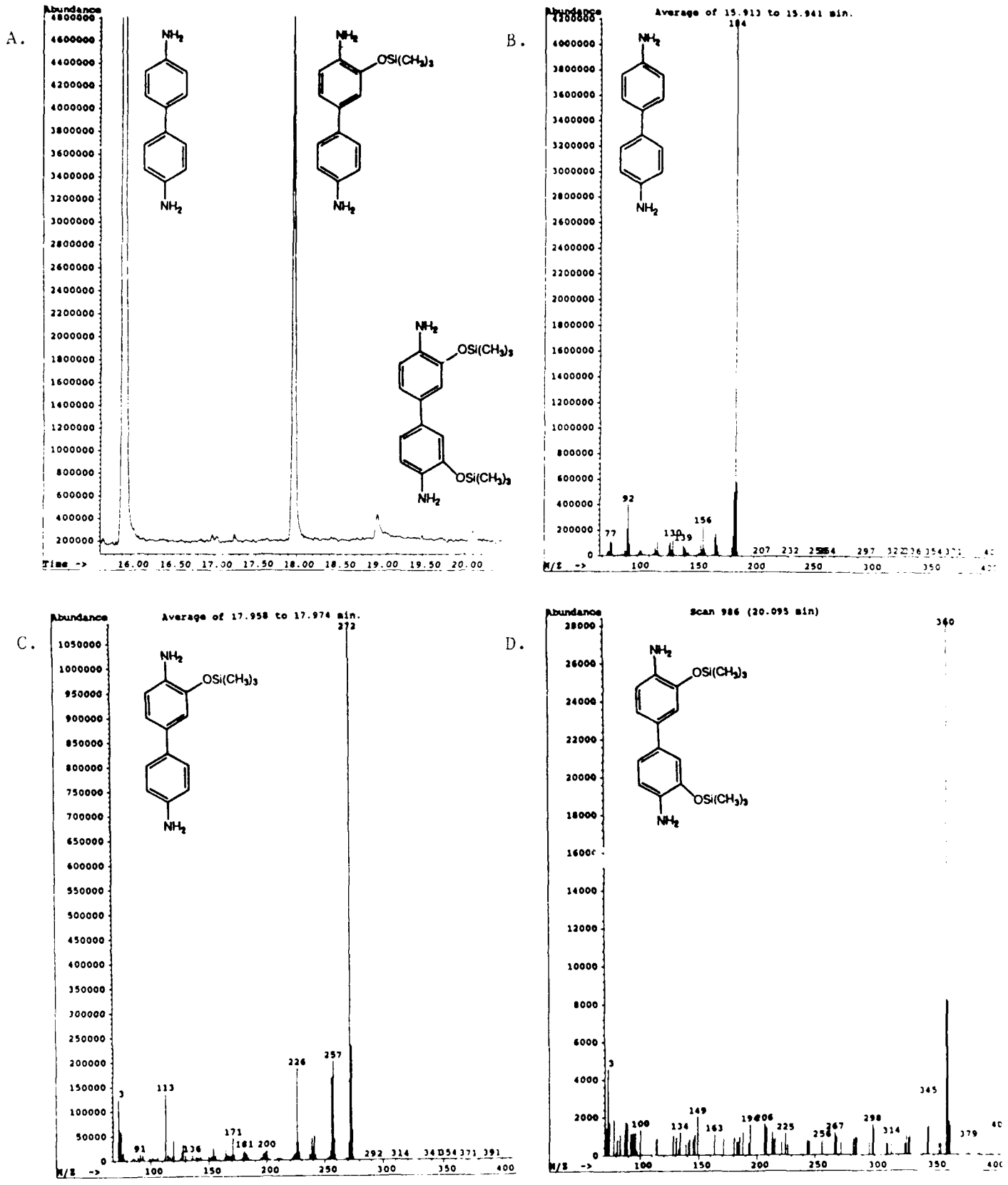


Figure 14. GC/MS analysis of a reaction between tyrosinase and benzidine. GC of the derivitized reaction mixture (A), and MS of compounds separated by GC (B-D).

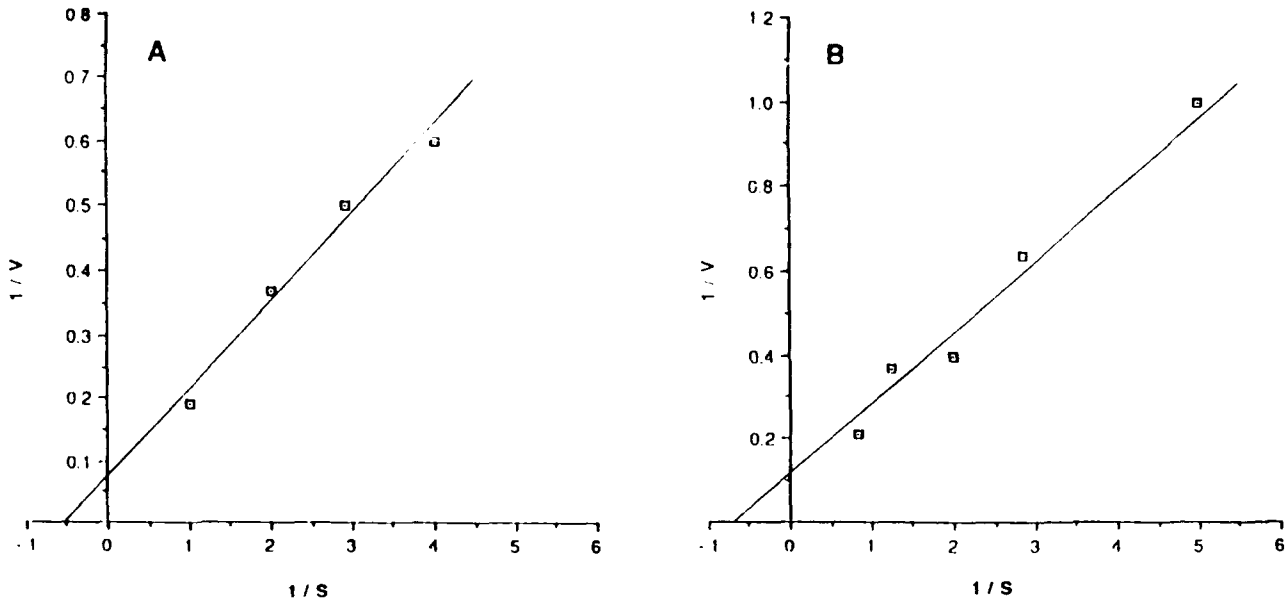


Figure 15. Lineweaver-Burk plots of data from the reaction of tyrosinase with bis(4-aminophenol)ether (A) or with benzidine (B). Plots represent the average of two assays. Substrate is expressed in mmole and reaction velocity is expressed as nmole O_2 uptake per minute per unit of enzyme.

The reaction of *Neurospora crassa* tyrosinase with two of these aromatic amines, benzidine and bis(4-aminophenyl)ether, was studied in more detail. These two substrates were chosen because the dihydroxylated products are interesting AA-type monomers for poly-benzoxazoles. The kinetic constants K_m and V_{max} are obtained from Lineweaver-Burk plots, shown in Figure 15. Assays contain 20 mM sodium phosphate, pH 6.5, 4 mM ascorbic acid, substrate, and enzyme in a volume of 3 ml. Assays are done at pH 6.5 to increase the solubility of these substrates, and even at this pH substrate concentration could not exceed 1.2 mM. Addition of ascorbic acid to the assay is necessary to observe significant activity. The reaction rates are determined from the measurement of oxygen consumption in an assay using the YSI oxygen electrode as described for the pH profile study. The K_m and V_{max} for benzidine under these conditions are 1.5 mM and 8.3 nmole oxygen/min/unit tyrosinase. The K_m and V_{max} for the bis(4-aminophenyl)ether are 1.8 mM and 13.3 nmole oxygen/min/unit

tyrosinase. The K_m values for benzidine and bis(4-aminophenyl)ether are near or just exceeding the solubility of these substrates, so the addition of a surfactant or organic solvent to increase the solubility of these compounds may be necessary to achieve maximum rates and conversions.

The ability of tyrosinase to hydroxylate 4'-aminobiphenyl-4-carboxylic acid was examined because the resulting hydroxylated product is an AB-type monomer with more oxidative stability than the corresponding dihydroxylated benzidine. Assays with 4'-amino-biphneyl-4-carboxylic acid were done at 30°C with *Neurospora* tyrosinase in the presence of ascorbic acid. Since benzoic acids are known to be inhibitory to tyrosinase activity, especially when the reaction pH is < 7.0 [32], the assays were done at pH of 7.5. The progress of the reaction was determined from the rate of oxygen consumption recorded with the YSI oxygen electrode. With 1 mM 4'-aminobiphenyl-4-carboxylic acid in the assay, 2.18 nmoles of oxygen were consumed/minute/unit of enzyme. As a comparison, with assays containing 1 mM benzidine at pH 6.5 under similar conditions, 2.61 nmoles of oxygen were consumed/minute/unit of enzyme. Thus, under these conditions the two substrates have similar reaction rates. The reaction rate with 4'-aminobiphenyl-4-carboxylic acid is very dependent on substrate concentration however, and falls off sharply at 1.2 mM and 0.8 mM substrate under identical conditions. This is probabaly due to the inhibitory nature of the carboxylic acid portion of the substrate.

4.B. 4-Hydroxybenzoate Hydroxylase.

4.B.1. Introduction

4-Hydroxybenzoate hydroxylase is a flavin-containing monooxygenase that catalyzes the hydroxylation of 4-hydroxybenzoic acid to 3,4-dihydroxybenzoic acid (Figure 16). This enzyme requires NADH of NADPH as the electron donor in the reation and incorporates one atom of molecular oxygen in the product. 4-Hydroxybenzoate hydroxylase has been isolated in pure form from several species of *Pseudomonas* (*P. flourescens* [33], *P. putida* [34], *P. desmolytica* [35], *P. testosteroni* [36] and *Corynebacterium cyclohexanicum* [37]) and the enzyme from *Pseudomonas* sp. is commercially available.

The mechanism of this three-substrate reaction has been investigated in detail [38, 39,40,41,42]. Initial velocity studies suggest a ping-pong type mechanism involving two ternary complexes between the enzyme and the substrates, as shown in Figure 17 [43]. Initially, NADPH and 4-hydroxybenzoic acid bind to the enzyme to form the first ternary complex, and this is followed by the release of the first product, $NADP^+$. The resulting complex now reacts with the third substrate, oxygen, to form the second ternary complex. The enzyme-bound 4-hydroxybenzoic acid and the activated oxygen in this complex then react, releasing 3,4-dihydroxybenzoic acid and regenerating the oxidized form of the enzyme.

One hypothesis for the structure of the activated oxygenating compound invokes addition of oxygen to the enzyme-bound FAD to form a 4a-hydroperoxyflavin [38]. This peroxide will then function as the oxygen transfer agent. The mechanism of this transfer involves nucleophilic attack of the ortho carbon of the phenolate on the peroxy oxygen, as shown in Figure 18.

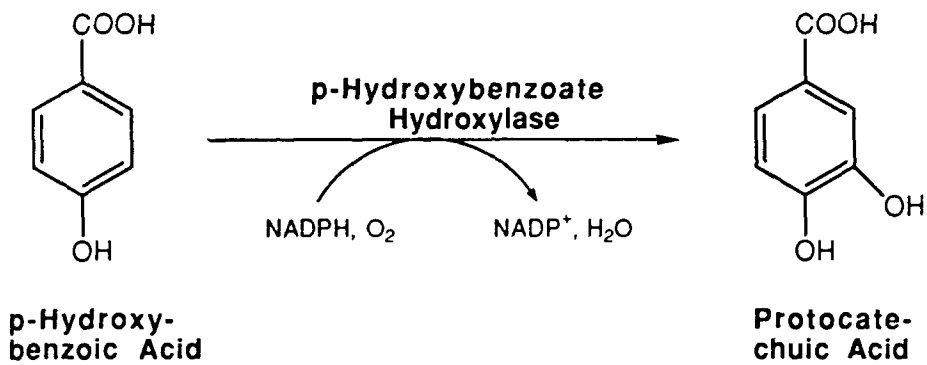


Figure 16. Reaction catalyzed by p-hydroxybenzoate hydroxylase.

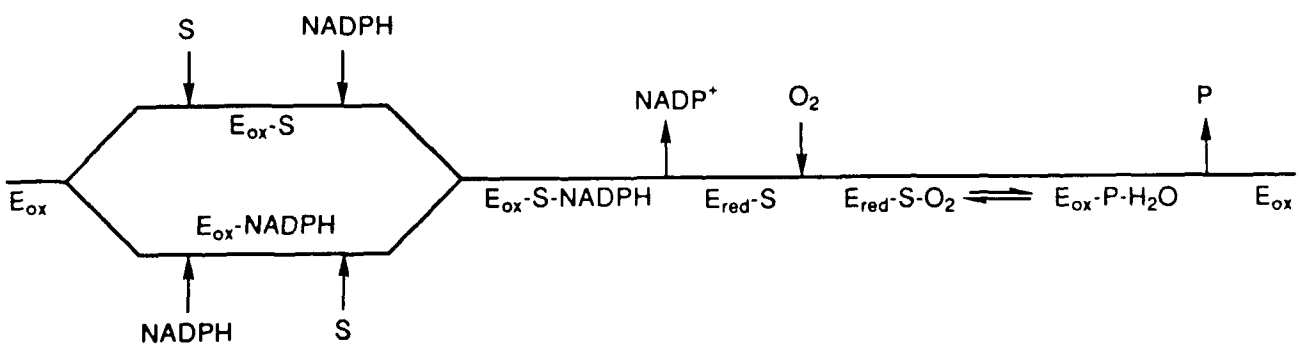


Figure 17. Ping-pong mechanism for the reaction catalyzed by p-hydroxybenzoate hydroxylase.

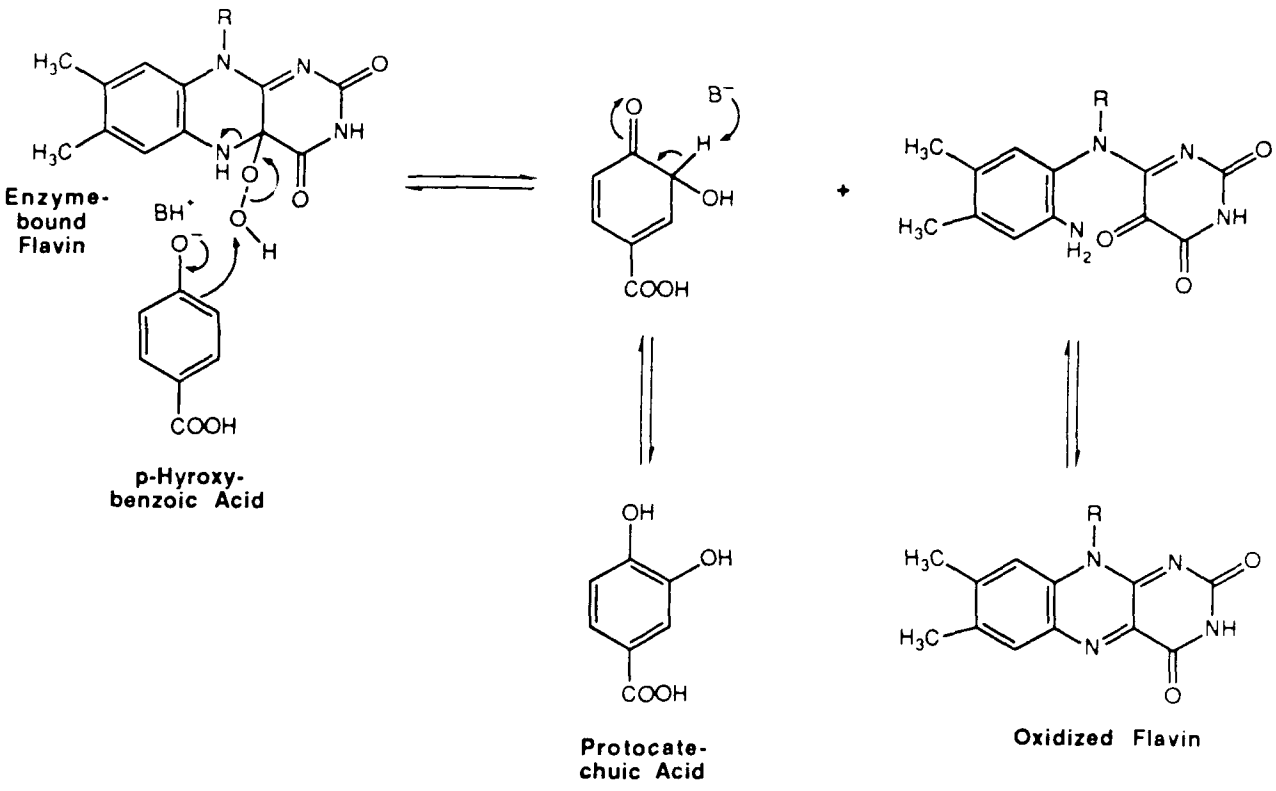


Figure 18. Proposed reaction mechanism for the reaction catalyzed by p-hydroxybenzoate hydroxylase.

Considering the proposed chemical reaction mechanism, 4-aminobenzoic acid may be a reasonable substrate analog for 4-hydroxybenzoate hydroxylase. The product of such a reaction would presumably be 4-amino-3-hydroxybenzoic acid, an AB-type polybenzoxazole monomer. However, steric constraints of the enzyme active site might impose limitations on the ability of the enzyme to hydroxylate alternative substrates, such as 4-aminobenzoic acid. For example, the 4-hydroxybenzoate hydroxylases isolated from *Corynebacterium cyclohexanicum* and *Pseudomonas putida* are highly specific for 4-hydroxybenzoic acid [34,37]. With these enzymes, 4-aminobenzoic acid is not hydroxylated, but it can act as an effector (i.e., increase the rate of NADPH consumed) [34,37].

In contrast to the 4-hydroxybenzoate hydroxylases from *C. cyclohexanicum* and *P. putida*, this enzyme isolated from *Pseudomonas fluorescens* has been reported to hydroxylate several substrates, including the hydroxylation of 4-aminobenzoic acid to produce 4-amino-3-hydroxybenzoic acid [38]. 4-Aminobenzoate hydroxylase isolated from *Pseudomonas testosteroni* also displays broad substrate specificity and will hydroxylate compounds such as 2-amino-3-hydroxybenzoic acid and 2-fluoro-5-hydroxybenzoic acid [36]. 4-Aminobenzoic acids have not been examined as substrates with this enzyme.

Based upon the proposed reaction mechanism and observed broad substrate specificity for *Pseudomonas* 4-aminobenzoate hydroxylase, this enzyme was studied to determine its usefulness as a catalyst in o-aminophenol monomer production.

4.B.2. Pre-contract Effort.

4-Hydroxybenzoate hydroxylase was isolated and purified from *Pseudomonas flourescens* grown on 4-hydroxybenzoic acid as its sole carbon source. This enzyme did catalyze the hydroxylation of 4-aminobenzoic acid to produce 4-amino-3-hydroxybenzoic acid, but at the very slow rate of $< 1\%$ of the rate of the reaction with 4-hydroxybenzoic acid as the substrate. Quantitative values for V_{max} and K_m with 4-aminobenzoic acid as substrate could not be obtained with the small amount of enzyme isolated.

In the enzymatic reaction, the hydroxylated products from 4-aminobenzoic acid and 4-hydroxybenzoic acid (4-amino-3-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid, respectively) were unstable in the reaction mixture. Concentrations of both these products decreased with time after the hydroxylation reaction was complete; no new products were observed by HPLC analysis with UV detection. The loss of products made the analysis of the reaction difficult and would certainly be undesirable if this reaction is to be used to produce 4-amino-3-hydroxybenzoic acid on a large scale. Since both 2-aminophenols and catechols are oxidatively unstable, ascorbic acid was added to the reaction in an effort to

staunch the presumed oxidative loss of products. The ascorbic acid successfully prevents the oxidation of 4-amino-3-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid for several days. This makes it possible to obtain good mass balances for the reactions and makes it easier to detect products from very slow reactions.

4.B.3. Results of Contract Effort

Effort was focused on studying the catalytic capabilities of a 4-hydroxybenzoate hydroxylase that is readily available from Calbiochem; this enzyme was purchased as a partially purified preparation (25.3 units/mg of solid) from a *Pseudomonas* species. A typical assay contains 0.5 mM substrate (either 4-aminobenzoic acid or 4-hydroxybenzoic acid), 0.5 mM ascorbic acid, 0.26 mM NADPH, and 0.4 mg of enzyme in 1 ml of 20 mM potassium phosphate at pH 8.1 and 23°C. The reaction rate is measured by monitoring absorbance of the reaction mixture at 340 nm; oxidation of NADPH results in a decrease in absorbance at 340 nm. The products, 4-amino-3-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid, are detected by HPLC analysis of the reaction mixtures, and their identities on the HPLC chromatogram are confirmed by co-injection with authentic samples of these compounds. In addition, the UV spectra of 4-amino-3-hydroxybenzoic acid produced by the enzyme is identical to that of a known sample of 4-amino-3-hydroxybenzoic acid. The UV spectra of both compounds shows absorbance maxima at 295 nm and 230 nm, and for both spectra the ratio of absorbance at 230 nm divided by the absorbance at 295 nm is 2.1. XX

With some oxidative enzymes containing flavin, hydroxylation of the substrate analog can be uncoupled from oxidation of NADPH. When this occurs, monitoring the oxidation of NADPH spectrophotometrically does not give meaningful data concerning the rate of hydroxylation of substrate. Therefore, before determining kinetic parameters for the hydroxylation of 4-aminobenzoic acid with 4-hydroxybenzoate hydroxylase, the reaction stoichiometry of the products ($NADP^+$ and 4-amino-3-hydroxybenzoic acid) must be determined. This is done by measuring the UV absorbance of the enzymatic reaction at 340

nm, the UV maximum for NADPH, and at 295 nm, the UV maximum for 4-amino-3-hydroxybenzoic acid. The extinction coefficients were measured at 340 nm and 295 nm for NADPH, $NADP^+$, 4-aminobenzoic acid (4ABA), and 4-amino-3-hydroxybenzoic acid (4A3HBA), and this information was used to derive the following equations:

$$dA340/dt = 6200(d[NADPH]/dt)$$

$$dA295/dt = 2000(d[NADPH/dt) + 300(d[NADP^+]/dt) + 3800(d[4ABA]/dt) + 7400(d[4A3HBA]/dt)$$

Since d[NADPH]/dt is equal to -d[$NADP^+$]/dt and d[4ABA]/dt is equal to -d[4A3HBA]/dt, this second equation simplifies to:

$$dA295/dt = 1700(d[NADPH]/dt) + 3600(d[4A3HBA]/dt)$$

By measuring the rate of change in absorbance at 295 nm and 340 nm in an enzyme assay and using these equations, the rate of NADPH oxidation can be compared to the rate of 4-amino-3-hydroxybenzoic acid production. The enzyme reactions in these experiments contained 0.12 mM 4-aminobenzoic acid, 0.10 mM NADPH, and 0.1 mg of enzyme in 50 mM potassium phosphate buffer at pH 6.5. With these experiments we found that the rate of NADPH oxidation is equal to the rate of 4-amino-3-hydroxybenzoic acid production. Thus, NADPH oxidation is not uncoupled from substrate hydroxylation.

The effect of pH on the enzyme reaction with both 4-hydroxybenzoic acid and 4-aminobenzoic acid was determined. Assays contain 0.5 mM ascorbic acid, 0.2 mM NADPH, 0.5 mM substrate and enzyme (0.005 mg for 4-hydroxybenzoic acid reactions and 0.4 mg for 4-aminobenzoic aicd reactions) in 1 ml of 50 mM buffer at 30°C. Initial rates are measured from the decrease in absorbance at 340 nm due to the oxidation of NADPH. Results are shown in Table 3. Note that the pH profile for the enzymatic reaction with 4-hydroxybenzoic acid varies significantly from the pH profile with 4-aminobenzoic acid as

the substrate. The pH optimum in the reaction with 4-hydroxybenzoic acid occurs at pH 8.1 while the pH optimum for the reaction with 4-aminobenzoic acid occurs at pH 6.0 to 6.5. There is no obvious explanation for the different pH optima measured with the two substrates since pK_a values for the amino acids in the enzyme active site that contribute to the binding of the substrate and pK_a values of the substrates are not near pH 6.0 to 8.0 [44].

4-Hydroxybenzoate hydroxylase purchased from Calbiochem was investigated in more detail to determine the V_{max} and K_m for the reaction with 4-hydroxybenzoic acid and 4-aminobenzoic acid. Enzyme assays contained 0.26 mM NADPH, 0.4 mg enzyme, and varying concentrations of substrate in 1 ml of 20 mM potassium phosphate at either pH 6.5 or 8.1. The reaction absorbance was monitored at 340 nm to determine the rate of NADPH oxidation, and the results are summarized in Table 4. The K_m value, a measure of the binding affinity of the enzyme for the substrate, does not change significantly when the pH of the reaction changes from 6.5 to 8.1. Furthermore, the K_m values for the two substrates are similar at both pH values indicating that the enzyme can bind 4-aminobenzoic acid nearly as well as 4-hydroxybenzoic acid. In contrast to the similarity of the K_m values, the V_{max} values do vary greatly for the two substrates. At pH 8.1, the rate of the reaction forr 4-aminobenzoic acid as measured by the oxidation of NADPH is 0.03% of the rate for 4-hydroxybenzoic acid. At pH 6.5, the rate of the reaction with 4-aminobenzoic aicd is approximately 5-fold faster than the rate at pH 8.1 with this substrate, and this corresponds to 0.41% of the rate with 4-hydroxybenzoic acid at pH 6.5.

The optimum temperature for the enzyme reaction is 45°C with either 4-hydroxy-benzoic acid or 4-aminobenzoic acid as the substrate, as shown in Table 5. The assays are done in 1 ml of 40 mM potassium phosphate at pH 6.5, 0.5 mM ascorbic acid, 0.2 mM NADPH, 0.5 mM substrate and 5 mg of enzyme. The assay mixture is preincubated at the desired temperature without the enzyme for at least 5 minutes. After addition of the enzyme, the rate of NADPH oxidation is determined from the change in absorbance at 340 nm. The enzyme also appears to be stable in solution at room temperature for a period of days.

Table 3. Effect of pH on p-hydroxybenzoate hydroxylase activity.

PP

Buffer	pH	4-Hydroxybenzoic Acid V_i(μmol NADPH/min/mg solid)	4-Aminobenzoic Acid V_i(μmol NADPH/min/mg solid)
Citrate	5	1.26	0.44×10^{-2}
Citrate	5.5	3.32	1.17×10^{-2}
Citrate	6	5.87	1.41×10^{-2}
Phosphate	6	5.48	2.42×10^{-2}
Phosphate	6.5	7.58	1.81×10^{-2}
Phosphate	7	8.19	1.17×10^{-2}
Phosphate	7.5	8.97	0.77×10^{-2}
Phosphate	8.1	10.80	0.45×10^{-2}

Table 4. Kinetic parameters of p-hydroxybenzoate hydroxylase.

pH	Substrate = 4-OH Benzoic Acid V_{max} (units/mg)	K_m (mM)	Substrate = 4-NH_2- Benzoic Acid V_{max} (units/mg)	K_m (mM)
6.5	4.1	2.5×10^{-2}	1.7×10^{-2}	7.8×10^{-2}
8.1	10.8	3.8×10^{-2}	3.6×10^{-3}	7.3×10^{-2}

Table 5. Temperature profile of p-hydroxybenzoate hydroxylase.

Temperature	4-Hydroxybenzoic Acid V_i (μmol NADPH/min/mg solid)	4-Aminobenzoic Acid V_i (μmol NADPH/min/mg solid)
30	7.7	2.01×10^{-2}
35	9.6	2.38×10^{-2}
40	10.6	2.64×10^{-2}
PP 45	12.1	2.86×10^{-2}
50	11.1	2.74×10^{-2}

This stability and the ability to function at higher temperatures are desirable properties for p-hydroxybenzoate hydroxylase. However, one serious limitation in large scale reactions with p-hydroxybenzoate hydroxylase is the requirement for NADPH. This limitation can be overcome if whole cells in a metabolically active state will catalyze the hydroxylation of 4-aminobenzoic acid. Two bacteria, *Pseudomonas aeruginasa* GS-1 and *Pseudomonas*-like bacterial strain designated MV1 (isolated and characterized at GE), are capable of growth on 4-hydroxybenzoic acid, presumably due to the presence of 4-hydroxybenzoate hydroxylase. *Pseudomonas aeruginosa* is reported to contain a 4-hydroxybenzoate hydroxylase that is nearly identical to the enzyme from *Pseudomonas fluorescens* [45], and the enzyme from *Pseudomonas fluorescens* is known to hydroxylate 4-aminobenzoic acid [38].

Pseudomonas aeruginosa GS-1 and bacterial strain MV1 were tested for their ability to catalyze the conversion of 4-aminobenzoate to 4-amino-3-hydroxybenzoate. *P. aeruginosa* GS-1 is not capable of growth on 4-aminobenzoic acid as the sole carbon source, and its growth on 4-hydroxybenzoic acid is inhibited in the presence of 4-aminobenzoic acid as deduced from the increase in doubling times. 4-Aminobenzoic acid is not hydroxylated to 4-amino-3-hydroxybenzoic acid when *P. aeruginosa* GS-1 is cultured in the presence of 4-aminobenzoic acid or with 4-hydroxybenzoic acid as the sole carbon source for growth. Furthermore, 4-aminobenzoic acid is not hydroxylated to 4-amino-3-hydroxybenzoic acid when late-log phase culture of *P. aeruginosa* GS-1 grown on 4-hydroxybenzoic acid is spiked with 4-aminobenzoic acid, or when late-log phase culture is centrifuged and the bacterial pellet is resuspended in minimal medium containing 4-aminobenzoic acid. Therefore, *P. aeruginosa* GS-1 is not a good catalyst for the synthesis of 4-amino-3-hydroxybenzoic acid.

Bacterial strain MV1, when grown to late-log phase on 4-hydroxybenzoic acid, harvested by centrifugation, and resuspended in minimal medium containing 4-aminobenzoic acid, does catalyze the conversion of 4-aminobenzoic acid to several other metabolites, one of which has an HPLC retention time and UV absorbance similar to 4-amino-3-hydroxy-

benzoic acid. However, since this is only a minor component of the reaction mixture, this route to monomer production is not very promising.

4.C Salicylate Hydroxylase

4.C.1 Introduction

Salicylate hydroxylase is a flavoenzyme that will oxidatively decarboxylate salicylic acid to produce catechol (Figure 19). This enzyme has been isolated from *Pseudomonas putida* [46,47], *Pseudomonas cepacia* [48], and *Trichosporon cutaneum* [49]. The salicylate hydroxylases isolated from these microorganisms differ in molecular weight, subunit structure, substrate specificity, and kinetic parameters. The *P. putida* enzyme is a monomeric protein of molecular weight 57,200 containing one FAD per molecule protein [46]; the *P. cepacia* enzyme is a dimeric protein of molecular weight 91,000 containing two FAD per molecule protein [48], and the enzyme from the soil yeast *Trichosporon cutaneum* is a monomeric protein of molecular weight 43,500 containing one FAD per molecule protein [49].

Kinetic analysis of salicylate hydroxylase indicates that both NADH and salicylate can form binary complexes with the oxidized enzyme, and the NAD^+ is released before oxygen binds [50,47] (Figure 20). This is consistent with a ping-pong mechanism similar to that of p-hydroxybenzoate hydroxylase. The order of release of the products, carbon dioxide and catechol, has not been determined.

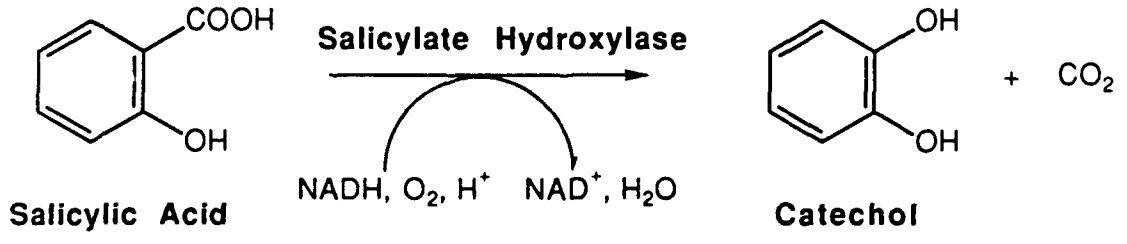


Figure 19. Reaction catalyzed by salicylate hydroxylase.

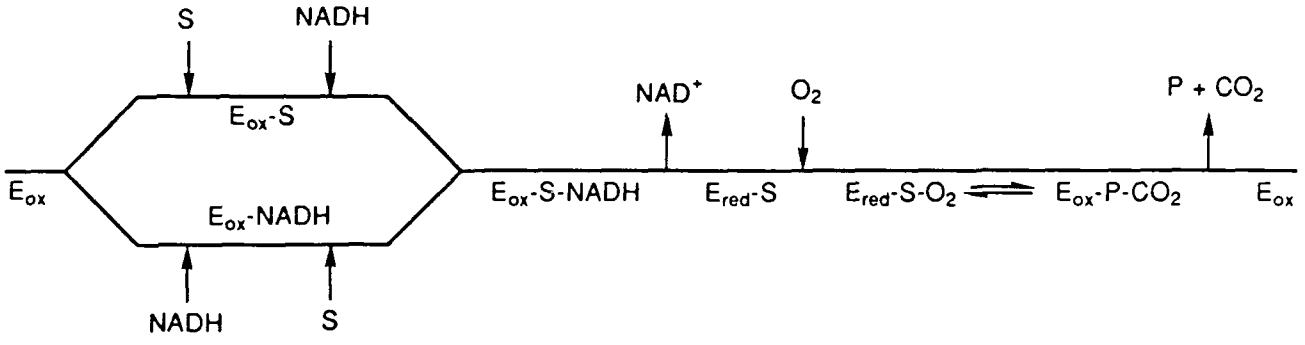


Figure 20. Ping-pong mechanism for salicylate hydroxylase.

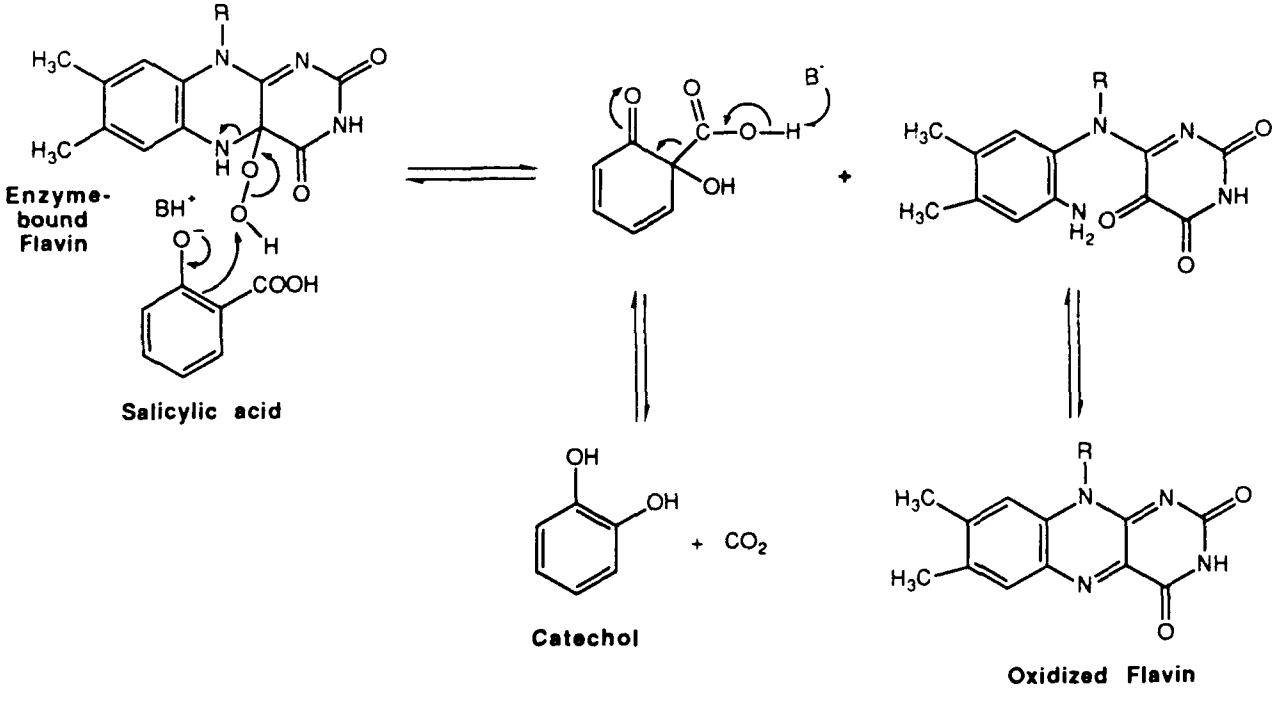


Figure 21. Proposed reaction mechanism for salicylate hydroxylase.

Presumable, the activated oxygen-transferring compound of this enzyme is similar to the 4a-hydroperoxyflavin hypothesized to be the oxygen-transferring compound of the p-hydroxybenzoate hydroxylase enzyme. A mechanism can be invoked that would be similar to the mechanism for p-hydroxybenzoate hydroxylase (Figure 21). Nucleophilic attack of the ortho carbon of the phenolate on the peroxy oxygen, followed by decarboxylation, would generate the product.

Salicylate hydroxylase, when isolated from *Pseudomonas cepacia* or *Trichosporon cutaneum*, displays activity toward a broad range of substrates [48,49]. Substituted salicylic acids (3-, 4-, 5-, or 6-position) are oxidatively decarboxylated, and with 4-hydroxysalicylic acid and 4-aminosalicylic acid the activity is enhanced by this substitution [48,49]. Since salicylate hydroxylase is active with substituted salicylic acid analogs, the active site geometry must properly orient the substrate even with additional substituents on the aromatic ring. In view of this and the proposed enzyme mechanism for the conversion of salicylate to catechol, we investigated the possibility that o-aminobenzoic acid (anthranilic acid) can substitute for salicylic acid in the reaction. Preliminary results in our laboratory indicated that a salicylate hydroxylase isolated from *Pseudomonas* sp. will accept anthranilic acid as a substrate with concomitant oxidation of NADH. The product of this reaction comigrates with o-aminophenol on the HPLC. With this encouraging result, we decided to investigate the extent to which substituted anthranilic acids will serve as substrates in this reaction.

4.C.2 Pre-contract Effort

A preliminary screening of the activity of salicylate hydroxylase with several analogs of salicylic acid was done to investigate the substrate specificity of this enzyme. Salicylate hydroxylase, isolated from *Pseudomonas* sp. strain ATCC 29352 and purchased from Sigma Chemical Company in a partially purified form, is assayed in 50 mM phosphate at pH 7.4 containing 0.5 mM DTT, 0.1 mM NADH, and varying concentrations of salicylic acid or substrate analogs. The reaction is followed by recording the depletion of NADH spectro-

Table 6. Substrate specificity of salicylate hydroxylase.

Substrate	K_m (μM)	V_{max} (μmol NADH/min/mg)	Relative Activity
Salicylic acid	1.84	0.392	100
3-Chlorosalicylate	4.09	0.042	11
4-Chlorosalicylate	5.18	0.495	126
5-Chlorosalicylate	1.23	0.052	13
Anthranilic acid	210	0.115	29

photometrically at 340 nm. NADH is oxidized to NAD^+ in the presence of the enzyme and substrate (salicylic acid, 3-chlorosalicylic acid, 4-chlorosalicylic acid, 5-chlorosalicylic acid, or anthranilic acid), and the K_m and V_{max} values for the reaction with these substrates is shown in Table 6.

Binding of 3-, 4-, or 5-chlorosalicylic acid to the enzyme is similar to the binding of the natural substrate as determined from the similarity in the K_m values. This result suggests that the binding site on the enzyme is not highly specific and accomodates additional substituents on the aromatic ring. As shown in Table 6, there is 100-fold difference in the K_m values for salicylic acid and anthranilic acid. Despite the large difference in K_m values for salicylic acid and anthranilic acid, the V_{max} for anthranilic acid is a respectable 30% of the V_{max} for the natural substrate when measured by the rate of NADH oxidation.

In addition to monitoring the reaction of NADH at 340 nm, the reaction solution is examined by HPLC to identify reaction products. Product cannot be identified in all cases despite the observation that NADH is oxidized to NAD^+. This could be due to intrinsic instability of the products in these solutions, as is the case for the reaction products of the 4-hydroxybenzoate hydroxylase reaction, or due to uncoupling of the enzyme reaction in

which a substrate analog binds to the active site in a fashion that promotes NADH oxidation without hydroxylation of the analog [51].

4.C.3. Results of Contract Effort

The rate and products of the reaction of salicylate hydroxylase with salicylic acid or anthranilic acid were examined in detail. For the reaction with salicylic acid, the assay contains 50 mM phosphate at pH 7.0, 0.1 mM DTT to stabilize the enzyme, 0.1 mM NADH, 0.1 mM salicylic acid, and the enzyme. The UV spectrum of NADH shows a maximum at 340 nm and the UV spectrum of salicylic acid shows a maximum at 295 nm. Therefore, the absorbance of the assay mixture is monitored at 340 nm and 295 nm, and the changes in absorbance are used to calculate the changes in concentration of these reactants. Under these conditions, the initial rate of the enzymatic reaction is 4.23 umol/min/mg protein for NADH oxidation and 4.02 umol/min/mg protein for salicylic acid oxidation. This suggests that NADH oxidation is closely coupled to catechol formation.

Under the above assay conditions with 0.1 mM anthranilic acid as the substrate, the initial rate of the enzymatic reaction is 0.19 umol/min/mg protein for NADH oxidation and 0.12 umol/min/mg protein for o-aminophenol production. This was calculated from the observed changes in absorbance at 282 nm (where the o-aminophenol has a UV maximum) and 350 nm (where NADH absorbs strongly and anthranilic acid absorbs weakly). The calculations took into account the changes due to absorbance of anthranilic acid, NAD^+, and NADH at 282 nm as well as the increase due to o-aminophenol production.

The data shows that salicylate hydroxylase does catalyze the oxidation of anthranilic acid to o-aminophenol. However, the rate of NADH oxidation with salycilic acid as the substrate is 20-fold greater than the rate of oxidation of NADH with anthranilic acid as the substrate. In addition, with anthranilic acid as the substrate, the oxidation of NADH is 1.5-fold faster than the rate of oxidation of anthranilic acid. This indicates that approximately 33% of the NADH oxidation in the reaction is due to uncoupling of the NADH oxidation

from anthranilic acid oxidation. This poses a serious problem when considering the use of this enzyme for large-scale production of o-aminophenol monomers. Recycling NAD^+ to NADH will be challenging enough without wasting 33% of the NADH due to uncoupling. The degree of uncoupling must be reduced to seriously consider salicylate hydroxylase as a catalyst for large-scale production of o-aminophenols.

At higher temperatures, the rate of the enzyme reaction may increase. In addition, the active site may change such that less uncoupling of the NADH oxidation from the anthranilic acid oxidation occurs. Assays of enzyme activity as a function of temperature were done with both salicylic acid and anthranilic acid as substrates. The assay conditions were the same as described above with the exception that the anthranilic acid concentration was increased to 0.2 mM. The results are summarized in Table 7. Salicylate hydroxylase has maximum activity at 40°C in the reaction with salicylic acid, and the maximum activity with anthanilic acid occurs at 30°C. The degree of uncoupling does not improve as the temperature is raised. Thus, raising the temperature has an overall deleterious effect on the activity of salicylate hydroxylase with anthranilic acid.

Table 7. Effect of temperature on salicylate hydroxylase activity.

Assay Temperature (°C)	Substrate = Salicylate (μmol/min/mg protein)		Substrate = Anthranilate (μmol/min/mg protein)	
	NADH	Salicylate	NADH	o-Aminophenol
30	5.05	5.20	0.55	0.38
35	5.88	5.62	0.62	0.31
40	6.53	6.23	0.52	0.30
47	5.78	6.15	0.45	0.27

Substrate and product inhibition were investigated with this enzyme. The enzyme was assayed with salicylic acid in the presence and absence of 0.1 mM or 0.5 mM o-aminophenol, and there was no significant difference in the initial rates of NADH oxidation in these three reactions. Likewise, if the enzyme was assayed with anthranilic acid as the substrate in the presence and absence of 0.1 mM or 0.5 mM o-aminophneol, there was no difference in initial rates of NADH oxidation. Thus, salicylate hydroxylase does not appear to be inhibited by o-aminophenol.

However, the rate of NADH oxidation in a reaction of salicylate hydroxylase and anthranilic acid does seem to depend on the anthranilic acid concentration. There is a 30% decrease in the initial rate of NADH oxidation when the enzymatic reaction contains 2.5 mM anthranilic acid instead of 0.5 mM anthranilic acid. Furthermore, if 0.02 units of enzyme is pre-incubated for 2 minutes with 0.5 mM anthranilic acid before addition of salicylic acid and NADH, there is a 50% decrease in initial rate of NADH oxidation over a reaction in which the enzyme was not pre-incubated with anthranilic acid. Pre-incubation of the enzyme with o-aminophenol causes no reduction in the initial rate of NADH oxidation when salicylic acid is the substrate. Thus, anthranilic acid seems to inhibit salicylate hydroxylase even at low concentrations, reducing the potential application of this enzyme for large scale production of o-aminophenol monomers.

The capability to synthesize o-aminophneol is a useful screening criteria for enzymes, but o-aminophenol is not a useful monomer for polybenzoxazoles. Therefore, salicylate hydroxylase was examined with aminoterephthalic acid as a substrate. This compound has the potential to be converted by the enzyme to 3-amino-4-hydroxybenzoic acid, an AB monomer for polybenzoxazoles. When salicylate hydroxylase is assayed with aminoterephthalic acid, there is no NADH oxidation. Thus, this analog of salicylic acid is not a substrate for the enzyme.

4.D. 4-Aminobenzoate Hydroxylase

4.D.1. Introduction

4-Aminobenzoate hydroxylase is an FAD-dependant monooxygenase that catalyzes the oxidative decarboxylation of 4-aminobenzoic acid to 4-aminophenol in the presence of NADH or NADPH and oxygen (Figure 22) [52]. This enzyme has been purified to homogeneity from the common edible mushroom *Agaricus bisporous* [53]. The enzyme consists of a single polypeptide of molecular weight 49,000 and exhibits a specific activity of 27.5 mmole NADH oxidized/min/mg protein [53]. The K_m values for 4-aminobenzoic acid, NADH and oxygen are 20.4, 13.6, and 200 mM, respectively. NADPH can serve as the electron donor in the reaction, but its K_m is 133 mM [53].

Although very little is known about the mechanism of this enzyme-catalyzed reaction, its similarity to the reaction catalyzed by salicylate hydroxylase suggests that it may catalyze the synthesis of o-aminophenols from substituted anthranilic acids. Indeed, anthranilic acid has been reported to be converted to o-aminophenol by this enzyme at 25% of the rate of the normal substrate [53]. Other compounds that bind to the enzyme and cause NADH oxidation include 4-aminosalicylic acid, 3,4-diaminobenzoic acid, and 4-amino-2-chlorobenzoic

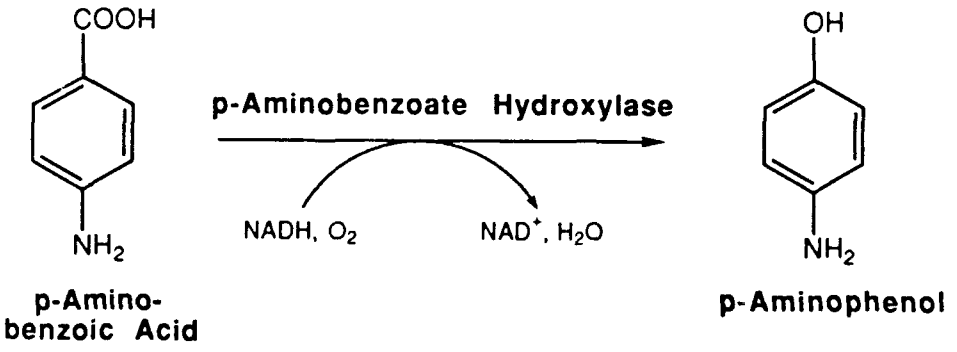


Figure 22. Reaction catalyzed by p-aminobenzoate hydroxylase.

acid [53]. Thus, 4-aminobenzoate hydroxylase displays a fairly broad substrate specificity and is a good candidate enzyme for the production of o-aminophenol monomers.

4.D.2. Pre-contract effort

None.

4.D.3. Results of Contract Effort

The protocol for the isolation of 4-aminobenzoate hydroxylase from mushrooms is very similar to that reported by Tsuji and co-workers [52]. Fresh mushrooms (1.1 kg) are washed in cold water, then homogenized at 4°C in a Waring blender with 2.2 L of buffer A (10 mM potassium phosphate at pH 7.1 containing 20% glycerol, 1 mM 2-mercaptoethanol, and 10 mM FAD). The homogenate is filtered through cheesecloth, and the filtrate is centrifuged at 9,500xg for 40 minutes to remove particulate matter. The supernatant is brought to 60% saturation with ammonium sulfate and centrifuged, and the pellet is suspended in buffer A. This is further fractionated by a second ammonium sulfate precipitation, and the pellet formed from 20-45% ammonium sulfate saturation is dissolved in a small amount of buffer B (50 mM potassium phosphate at pH 7.1 containing 10 mM 2-mercaptoethanol). After dialysis against buffer A, the dialysate is passed through a Sephadex G-100 gel filtration column equilibrated in buffer A. The enzyme is eluted with the same buffer, and fractions containing the enzyme activity are pooled. The volume is reduced by ultrafiltration, and the concentrated enzyme solution is stored at -20°C.

This crude enzyme preparation is further purified by anion exchange chromatography using a Mono-Q anion exchange column on a Pharmacia FPLC. The enzyme is eluted with a gradient of NaCl in 25 mM Tris buffer, pH 7.2. Fractions containing the highest activity are pooled to achieve a preparation consisting of 1.0 mg protein with a specific activity of 0.22 units/mg (a unit is defined as mmole NADH oxidized per minute). Flanking fractions are also pooled to achieve a second preparation consisting of 1.4 mg protein with a specific

activity of 0.092 units/mg. Interestingly, a small amount of activity always eluted in the void volume suggesting that this enzymatic activity may exist in other proteins within the mushroom. A summary of the purification is shown in Table 8. Although the total units isolated from the mushrooms are lower than that reported by Tsuji and co-workers [52], the specific activity and yield compare favorably with their results.

4-Aminobenzoate hydroxylase purified to homogeneity has a specific activity of 27.5 units/mg [53]. The enzyme preparation described above has a specific activity of 0.22 units/mfg and thus is still very impure. Before critically evaluating the ability of this enzyme to serve as a catalyst for polybenzoxazole monomer production, the enzyme preparation had to be examined to ascertain whether the impurities would cause interference in the 4-aminobenzoate hydroxylase reaction. In particular, the enzyme preparation could have been contaminated with tyrosinase which catalyzes the oxidation of o-aminophenol to a yellow product with maximum absorbance at 410 nm.

Table 8. Purification of p-aminobenzoate hydroxylase.

Enzyme assays were done at 25°C as described by Tsuji et al. [52]. Protein assays were done by the BioRad protein stain protocol. (1 Unit = 1 μmol 4-aminophenol/min).

	Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield (%)	Purification (-fold)
Clarified Homogenate	5,456	7.9	0.00144	100	1
$(NH_4)_2SO_4$ (0-60%)	2,520	15.1	0.00597	191	4
$(NH_4)_2SO_4$ (20-45%)	1,628	14.5	0.00891	183	6
Seph. G-100	88	4.2	0.0477	53	33
Mono-Q	1	0.22	0.22	3	153

To determine whether o-aminophenol was stable under standard assay conditions, o-aminophenol in a final concentration of 0.2 mM was combined with 50 mM potassium phosphate, pH 7.0, 0.02% bovine serum albumin (BSA), 0.2 mM NADH, 0.1 mM FAD, and 2.2 ug enzyme preparation. This assay was incubated at 30°C for 15 minutes with continuous monitoring of absorbance at 282 nm, where o-aminophenol has a maximum absorbance, and at 410 nm. There was no significant decrease in the absorbance at 282 nm or increase in the absorbance at 410 nm. Therefore, this enzyme preparation did not contain contaminants which react with o-aminophenol, and we proceeded to examine the effect of pH on enzyme activity and the subatrate specificity of this enzyme preparation.

This preparation of 4-aminobenzoate hydroxylase was assayed with both 2- and 4-aminobenzoic acid at pH values ranging from 5.0 to 9.0. The assays contain 50 mM buffer, 0.2 mM NADH, 0.05 mM FAD, 0.02% BSA, 1 mM substrate, and 2.2 to 4.4 mg protein from the enzyme preparation. The buffers are either citrate (pH 5.0 to 6.0), potassium phosphate (pH 6.0 to 8.0) or Tris-HCl (pH 7.5 to 9.0). The reactions are done at 30°C and followed spectrophotometrically at 350 nm to monitor the rate of NADH oxidation. Figure 23 shows the pH profile generated from this data. Note that although the specific activity is lower for the 2-aminobenzoic acid than for the 4-aminobenzoic acid, this enzyme has essentailly the same pH profile for both the natural substrate and the substrate analog. This is in contrast to the results with 4-hydroxybenzoate hydroxylase and suggests that the mechanism of catalysis by 4-aminobenzoate hydroxyalse is the same for both substrates.

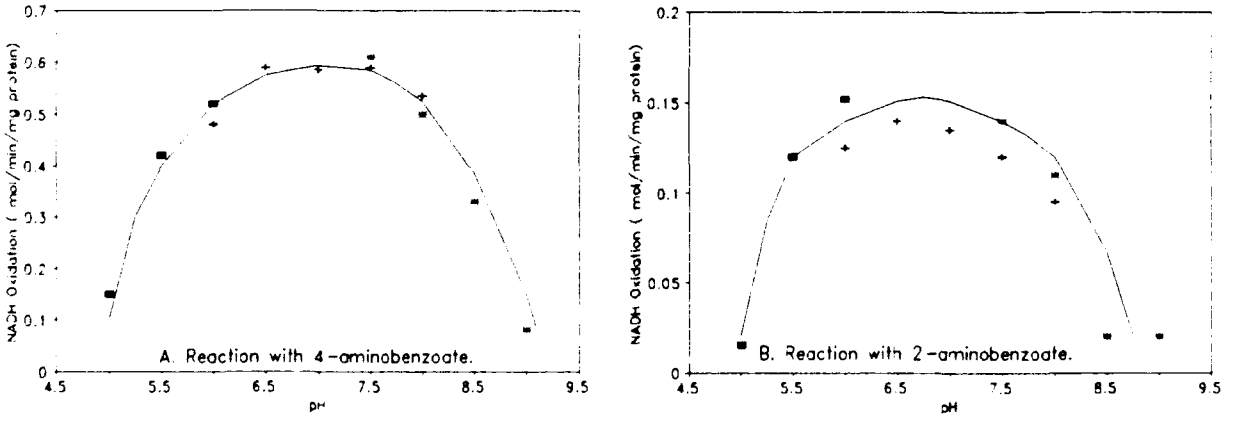


Figure 23. Effect of pH on p-aminobenzoate hydroxylase activity.

In the presence of certain substrate analogs, 4-aminobenzoate hydroxylase can catalyze the oxidation of NADH to NAD^+ without concomitant decarboxylation and hydroxylation of the substrate analog. This process, refered to as uncoupling, produces hydrogen peroxide. Due to this uncoupling, merely monitoring NADH oxidation in an assay spectrophotometrically does not necessarily quantify product formation. Therefore, several experimental approaches were examined to determine the best assay for measuring enzyme activity towards substrate analogs. The approaches examined were: (1) spectrophotometric determination of substrate and/or product concentrations, (2) measurement of oxygen concentration with a YSI oxygen electrode, and (3) separation and quantitation of substrates and/or products by HPLC. The results of this examination are reported below.

The initial substrates to be analyzed and the expected products are listed in Table 9. As shown in Table 9, 4-aminobenzoic acid, 4-aminophenol, 2-aminobenzoic acid, 2-aminophenol, and 3-amino-4-hydroxybenzoic acid have maximum absorbances in a region remote from the 340 nm maximum for NADH. Thus, NADH concentration can be monitored spectrophotometrically for the 4-aminobenzoate hydroxylase assays of 2- and 4-aminobenzoic acid. However, aminoterephthalic acid has a maximum absorbance at 330 nm and thus interferes significantly with the spectrophotometric determination of NADH concentration. In addition, the region of maximum absorbance for many of the products (270 nm to 290 nm) has interferring absorbance due to the FAD in the assay. This high background absorbance decreases the sensitivity towards changes in absorbance in this region, and thus product formation cannot be quantitated accurately. Therefore, this experimantal approach is useful only for monitoring NADH concentrations for certain substrates in assays of 4-aminobenzoate hydroxylase.

The second approach to assay the activity of 4-aminobenzoate hydroxylase involved the constant monitoring of oxygen concentration under standard assay conditions, followed by addition of catalase at the end of the reaction to determine the amount of hydrogen peroxide produced. Unfortunately, this method used by Tsuji et al. [53] to determine the

Table 9. UV spectroscopic properties of substrates and predicted products for p-hydroxybenzoate hydroxylase.

Substrate	λ_{max}	ϵ	Product	λ_{max}	ϵ
4-Aminobenzoic acid	285 nm	12,730	4-Aminophenol	290 nm	2,100
2-Aminobenzoic acid	310 nm	2,800	2 Aminophenol	280 nm	2,510
Aminoterephthalic acid	330 nm	2,970	3-Amino-4-hydroxy-benzoic acid	300 nm	3,520
NADH	340 nm	6,220	NAD^+	259 nm	16,700

degree of uncoupling that occurred with substrate analogs, proved to be an invalid measure of uncoupling due to the instability of hydrogen peroxide in our reaction conditions.

The third approach to assay the enzyme activity involved the use of paired-ion chromatography on HPLC (PIC-HPLC) to separate and quantify substrates and/or products of the assay. Aliquots of an assay mixture as a function of time were examined by PIC-HPLC and the products and substrates were separated and quantitated from peak areas. The column used in this analysis was an analytical Whatman PartiSphere C18 column. The substrates and products were eluted from the column in a linear gradient of 100% eluent A (water containing 50 mM tetrabutylammonium dihydrogen phosphate) to 100% eluent B (eluent A containing 80% acetonitrile) in 15 minutes at a flow rate of 2 ml/minute. Substrates and products were detected at 250 nm, 260 nm, 270 nm, or 290 nm (depending on the substrate analogs being examined), and 340 nm.

The substrate specificity of 4-aminobenzoate hydroxylase was examined by using the PIC-HPLC enzyme assay with a number of substrate analogs. Both the rate of NADH oxidation and the degree of uncoupling were determined for each substrate examined. The data obtained from these assays is shown in Table 10. When 2-aminobenzoic acid is the substrate, the specific activity is an encouraging 30% that of the natural substrate,

4-aminobenzoic acid, and NADH oxidation is 22% uncoupled from production of 2-aminophenol. This is in good agreement with the findings of Tsuji et al. [53]. However, 2-aminophenol is not a useful polybenzoxazole monomer, and with all other substrate analogs examined, the NADH oxidation catalyzed by this enzyme was completely uncoupled from product synthesis. Thus, 4-aminobenzoate hydroxylase displays a substrate specificity that is incompatible with its use as a catalyst for the synthesis of polybenzoxazole monomers.

Table 10. Substrate specificity of p-aminobenzoate hydroxylase.

Substrate	NAD^+ Synthesis (μmol/min/mg protein)	% Uncoupling
4-Aminobenzoic acid	0.444	13
2-Aminobenzoic acid	0.132	22
3-Aminophthalic acid	0.025	100
Aminoterephthalic acid	0.010	100
4-Nitrobenzoic acid	0.024	100
2-Nitrobenzoic acid	0.029	100
5-Methyl-2-aminobenzoic acid	0.129	100

4.E. Reduction of o-Nitrophenols Catalyzed by Bacteria

4.E.1. Introduction

The reduction of nitroaromatic compounds to the corresponding aminoaromatic compounds is catalyzed by a variety of microorganisms under anaerobic conditions [12-15,54]. Although the detailed enzymology for these reductions is not well understood, a number of observations have been made concerning these reactions. First, the ability to reduce nitro groups on aromatic rings under anaerobic conditions has been documented for many microorganisms including *Pseudomonas denitrificans*, *Pseudomonas fluorescens*, *Escherichia coli*, *Veillonella alkalescens*, *Neurospora crassa*, *Aspergillus niger*, *Nocardia V*, and *Clostridium pasteurianum* [12-15,54,55,56,57]. Second, the substrate specificity appears to be very broad and includes mononitro-, dinitro-, and trinitro- nitrotoluenes, benzenes and benzoic acids [54]. The reactivity of the nitro group in these substrates depends on the other substituents on the ring and on the position of these substituents. For example, in the para position, electron withdrawing substituents increase the rate of reduction of the nitro group; thus, the rate of reduction for a series of nitrobenzenes substituted in the para position is $NO_2 > CO_2H > CH_3 > H > OH > NH_2$ [54]. Third, the resulting aminoaroamtic compounds are stable in the presence of these bacterial catalysts under anaerobic conditions. Under aerobic conditions, the aminoaromatic compounds are frequently further metabolized.

Nitro-reductases from a few organisms have been at least partially purified and then studied [54-57]. With the strict anaerobe *Veillonella alkalescens*, the nitro-reductase seems to consist essentially of hydrogenase and a ferrodoxin-like material that can be separated from each other by Sephadex G-200 chromatography [54]. The reduction proceeds through the following sequence of reactions:

$$R\text{-}NO_2 \xrightarrow{H_2} R\text{-}NO \xrightarrow{H_2} R\text{-}NHOH \xrightarrow{H_2} R\text{-}NH_2$$

In the reduction, 3 moles of hydrogen are utilized for every mole of nitro group that is reduced. This nitro-reductase has been used to catalyze the reduction of 40 mono-, di-, and tri- nitroaromatic compounds. The nitro-reductase from *Nocardia* V has also been partially purified [55]. Like that from *Veillonella alkalescens*, this nitro-reductase appears to consist of a single protein complex capable of carrying out the complete reduction of aromatic nitro groups to the corresponding amino groups. The purified nitro-reductases are not stable and must be stored frozen to prolong activity.

Because of the ease with which a variety of microorganisms can catalyze the reduction of nitroaromatic compounds and the broad substrate specificity of these reactions, a promising approach to the synthesis of polybenzoxazole monomers is chemical nitration of a phenol to yield o-nitrophenol and then biological reduction of the nitrophenol. Advantages of this approach include: (1) production of the oxidatively unstable o-aminophenol monomer under oxygen-free conditions that maintains stability of the desired product, (2) ability to obtain high substrate and product concentrations due to tolerance of the bacteria to these compounds, (3) mild reaction conditions (aqueous solution at low temperatures) which eliminates the need for organic solvents, and (4) simple product recovery due to the quantitative conversion of the o-nitrophenol to the o-aminophenol under anaerobic conditions.

4.E.2. Pre-contract Effort

The GE Biological Sciences Laboratory has several bacterial cultures, mixed and pure, that actively reduce nitrates, nitrites, and nitroaromatic compounds. These cultures originate from the mixed culture of a waste water treatment facility at a GE plastics manufacturing plant. This biomass was used in an enrichment for growth on phthalimide as sole source of carbon and energy in a minimal media. The bacteria able to metabolize phthalimide were further enriched for nitro-reductase activity by incubation under anaerobic conditions with nitroaromatic compounds such as 3-nitro-N-methylphthalimide.

4.E.3. Results of Contract Effort

We first took advantage of the ability to biologically reduce aromatic nitro groups in the synthesis of 4'-aminobiphenyl-4-carboxylic acid, a potentially interesting substrate for tyrosinase which yields 4'-amino-3'-hydroxybiphenyl-4-carboxylic acid when hydroxylated. The synthesis of 4'-aminobiphenyl-4-carboxylic acid is shown in Figure 24. Biphenyl-4-carboxylic acid **1** is nitrated to yield 4'-nitrobiphenyl-4-carboxylic acid **2**. By incubating **2** under anaerobic conditions in the presence of bacterial culture PI3A, a GE bacterial isolate, **2** is reduced to 4'-aminobiphenyl-4-carboxylic acid **3** at a final concentration of approximately 8 g/L. The bacterial culture remains viable and active throughout this reduction. There are no UV-detectable products from **2** other than **3** at the completion of the reaction, and the supernatant of the culture contains no other UV-detectable compounds in significant quantities. Complete recovery of **3** is accomplished by adjusting the pH of the solution to 4.5 and then collecting the resulting precipitate. After recrystallization, 4'-aminobiphenyl-4-carboxylic acid is pure as determined by HPLC analysis using detection at 220 nm to 350 nm with a photodiode array detector and by NMR. The structure of **3** is confirmed by NMR analysis and by mass spectroscopy.

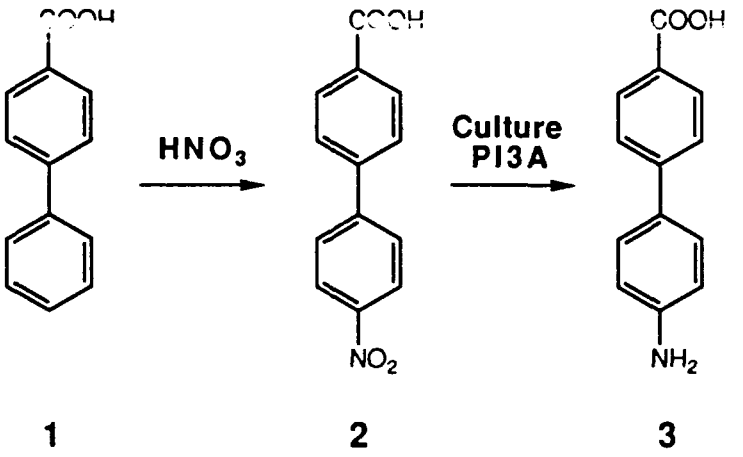


Figure 24. Bio/chemical synthesis of 4'-aminobiphenyl-4-carboxylic acid.

The ability to catalyze the reduction of aromatic nitro groups is common to many microorganisms. Therefore, we did an experiment to determine the nitro-reductase activity of several microorganisms in an attempt to narrow our focus to just one culture for further study. The cultures examined in this experiment were: (1) SL1, a mixed culture derived from a glucose-fed anoxic batch reactor containing nitroaromatic compounds, (2) SL2 and SL3, unidentified pure bacterial cultures isolated from SL1, (3) *Pseudomonas denitrificans* ATCC 13867 and ATCC 19244, (4) EU1, a mixed culture derived from a batch reactor which contained nitroaromatic compounds and corn steep liquor, and (5) PI3A, a co-culture of unidentified bacteria and PI1, a GE bacterial isolate selected from a mixed culture exposed to nitroaromatic compounds. These cultures were incubated anaerobically with a nitroaromatic substrate (4-nitro-N-butylphthalimide) in a minimal media containing either yeast extract or glucose. With the exception of *Pseudomonas denitrificans* ATCC 19244, all cultures examined were capable of reducing this nitroaromatic substrate. The data for the reduction of base-hydrolized 4-nitro-N-butylphthalimide is presented in Table 11. Since these experiments were done with whole cells at varying cell densities, the rates of reduction have been normalized to a cell density corresponding to 1 AU at 610 nm.

Table 11. Rate of aromatic nitro group reduction catalyzed by different anaerobic cultures.
n.d. = not determined.

Culture	Aromatic Nitro Group Reduction (ppm/hr/1 AU cells at 610 nm)	
	Yeast Extract	Glucose
SL1	60	50,70
EU1	50	6
SL2	35	33
SL3	31	n.d.
ATCC 13867	46	31
P13A	350	n.d.

As shown in Table 11, the greatest rate of reduction under these conditions was obtained with PI3A. Therefore, this culture was examined further to determine its utility as a catalyst for the reduction of nitrophenols. The experiments with PI3A culture are typically done by inoculating minimal media (40 mM sodium phosphate, pH 8.5, containing 40 mM ammonium chloride, 0.4% yeast extract, 500 ppm of 4-hydroxy-3-nitrobenzoic acid, and trace metals) in a serum bottle with PI3A culture to an initial AU of < 0.1 at 590 nm. The bottle is sealed and incubated at 30°C with shaking; the culture grows aerobically until the oxygen is depleted, and then growth and reduction of the nitrophenol occurs under anaerobic conditions. Reduction of 4-hydroxy-3-nitrobenzoic acid is quantified by HPLC analysis of the supernatant of the culture. The HPLC analysis involves elution of the sample from a C_{18} column with a linear gradient from water containing 0.1% trifluoroacetic acid and 0.05% triethylamine to acetonitrile containing 0.05% triethylamine.

The PI3A culture contains bacteria of three predominant phenotypes when plated on solid LB media (per liter, 10 g bactotryptone, 5 g NaCl, 5 g yeast extract, and 1.5 g agar). These phenotypes are: white, raised, compact colonies; yellow, raised, compact colonies; and transluscent, spreading colonies. When another PI3A culture that actively reduced nitro-aromatic compounds was plated on LB media, the resulting colonies were either one of the two phenotypes seen earlier (white and yellow raised colonies) or a new third phenotype of pink, raised colonies. In all experiments with PI3A cultures that catalyzed the reduction of nitrophenols, both the yellow and white raised colonies are present when the cultures are plated on LB plates, and generally at least one other phenotype is present as well.

Bacterial colonies of the various phenotypes were purified in an attempt to identify the bacteria responsible for the nitro-reductase activity. Since the bacteria are all facultative anaerobes, the purification is accomplished by repeated isolation of individual colonies, aerobic growth of these isolates in liquid minimal media, and plating of the resulting culture on LB media. The ensueing purified bacteria were then used to inoculate minimal media containing 4-hydroxy-3-nitrobenzoic acid, and the ability of the individual isolates to reduce this substrate anaerobically was determined. For the isolates with the transluscent, spreading

colony morphology (designated strain PI3) and the pink, raised colony morphology (designated strain PI2), no nitro-reductase activity is obtained in pure cultures. With the isolates having a white, raised colony morphology and yellow, raised colony morphology, reduction of the nitrophenol occurrs after a lag period of several days. These pure cultures were plated on LB media after the onset of nitro-reductase activity, and both cultures had the two colony morphologies (white and yellow raised colonies). Apparently, these two morphologies must be present to have detectable nitro-reductase activity.

The isolates with the white, raised colony phenotype, designated strain PI1W, and the yellow, raised colony phenotype, designated strain PI1Y, were submitted to the American Type Culture Collection (ATCC) for characterization. The results of this analysis, shown in Appendix A, strongly suggests that these strains are the same bacterium manifesting two different phenotypes when grown aerobically on solid LB media. PI1 is a gram positive, non-motile bacterium identified as Group D Streptococci. The cells of this bacterium grow in chains, and growth occurs over a temperature range of 10°C to 45°C and in up to 6.5% NaCl. We found that although the rate of growth in minimal media is greater at 30°C than at 26°C, the rate of nitroaromatic reduction after the culture had grown to 1.2 AU_{590} is not significantly different. The reduction was not studied at elevated temperatures; the effect of temperature on the reduction rate needs to be better defined.

The onset of nitro-reductase activity is faster when strains PI1 and PI2 are cultured together anaerobically in minimal media containing a nitrophenol and 0.4% yeast extract as the carbon source. PI2 is a gram-negative, facultative anaerobe that has not been fully characterized. PI2 was examined with Biolog microplates (Biolog, Inc., Hayward, CA), which test the ability of a microorganism to oxidize a preselected panel of 95 different carbon sources; in wells that contain a chemical that is oxidized, there is a burst of respiration and the cells reduce a tetrazolium dye forming a purple color. The results (Appendix B) are compared to a computerized data base to determine the best match with the responses from bacteria with known genera and species designations. PI2 does not match well with any of

the bacteria in the data base, and so remains unidentified. The role that it plays in the co-culture with PI1 needs to be further elucidated.

The co-culture of PI1 and PI2 was used to examine the range of nitroaromatic compounds that could be reduced. These reactions typically are done in serum bottles filled at least 80% with the minimal media containing 0.4% yeast extract and 500 ppm of the nitroaromatic substrate. After inoculation with a co-culture of PI1 and PI2, the bottles are sealed and incubated at 30°C with shaking. The pH is maintained between 8.0 and 8.5 by the addition of base to the culture when needed. The progress of the reaction is monitored by removing aliquots from the reaction as a function of time and analyzing the supernatant of these samples by HPLC to quantitate the substrate and/or product. Often, transient intermediates are detected by this HPLC analysis (Figure 25); although we speculate that these may be a partially reduced form of the nitroaromatic compound, these have not yet been positively identified. After the reduction is complete, the desired aromatic amine can be extracted from the bacterial culture. If the resulting o-aminophenol is oxidatively unstable, then the monomer can be first derivitized by the addition of acetic anhydride to the aqueous culture to acetylate the amino group. The acetylated o-aminophenol can then be extracted from the culture, recrystallized, and examined by NMR, HPLC, and mass spectroscopy for structure verification and purity determination.

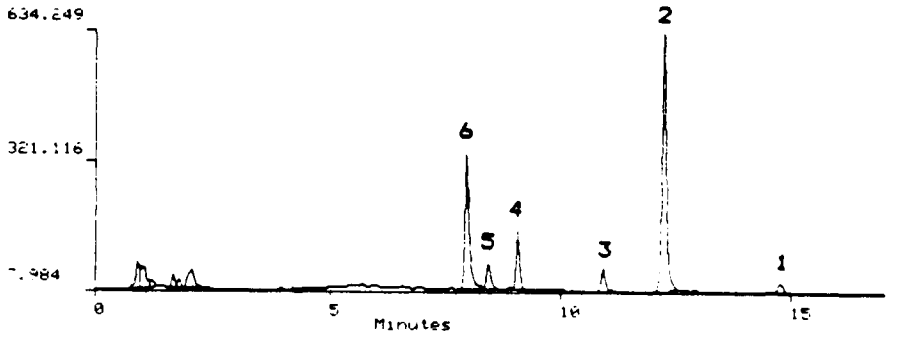


Figure 25. HPLC analysis of the supernatant from a co-culture of PI1/PI2 and 3,3″-dinitro-m-terphenyl-4,4″-diol. Peak 1 corresponds to the nitroaromatic substrate. Peaks 2-5 occur transiently during the reduction reaction. Peak 6 accumulates during the reduction and is presumably the fully reduced product.

The nitroaromatic substrates reduced by the PI1/PI2 co-culture include: 4-hydroxy-3-nitrobenzoic acid, 3-hydroxy-4-nitrobenzoic acid, o-nitrophenol, 4-nitrobenzoic acid, 2,4-dinitrotoluene, 3,4′-dinitrobiphenyl-4-carboxylic acid, 4′-nitrobiphenyl-4-carboxylic acid, 4,6-dinitroresorcinol, and 2,2-bis(4-hydroxy-3-nitrophenyl)propane (Figure 26). For most of these substrates, there were multiple additions of the substrate to the culture in an attempt to increase the concentration of product while keeping the concentration of the nitrophenol moderate. This enables us to determine the tolerance level of the culture to high product concentrations and to synthesize enough product for its isolation and characterization. Typically, at least 1 g/L of substrate is reduced in an experiment, and concentrations as high as 8 g/L of 4′-aminobiphenyl-4-carboxylic acid and 4-hydroxy-3-aminobenzoic acid were reduced in a co-culture of P1/P2. HPLC analysis of these reaction mixtures at the completion of the reduction characteristically show only one peak that is not due to the compounds present in the growth media; this peak has a retention time shorter than that of the substrate, consistent with identification of this peak as reduced product. Under anaerobic conditions, the products are not further metabolized by this culture. Therefore, there is approximately 100% conversion of substrate to product in these cultures.

The average rates of reduction for the substrates listed in Figure 26 are 20 ppm/hr/1 AU_{590}, with rates as high as 30 ppm/hr/1 AU_{590} obtained under some conditions. These rates seem low when compared to the 350 ppm/hr/1 AU_{590} reduction rate of base-hydrolized 3-nitro-N-butylphthalimide (see Table 11). However, the rate may be substrate-dependant (PI3A culture was enriched on base-hydrolized 3-nitro-N-methylphthalimide, so the rate of reduction may be greater with this substrate than with other nitroaromatic compounds). In addition, the amount of strain PI1 in these mixed cultures is not likely to be the same, so normalizing to a bacterial density corresponding to 1 AU at 590 nm introduces error. These rates can only be improved upon as more optimum conditions for these reductions are defined.

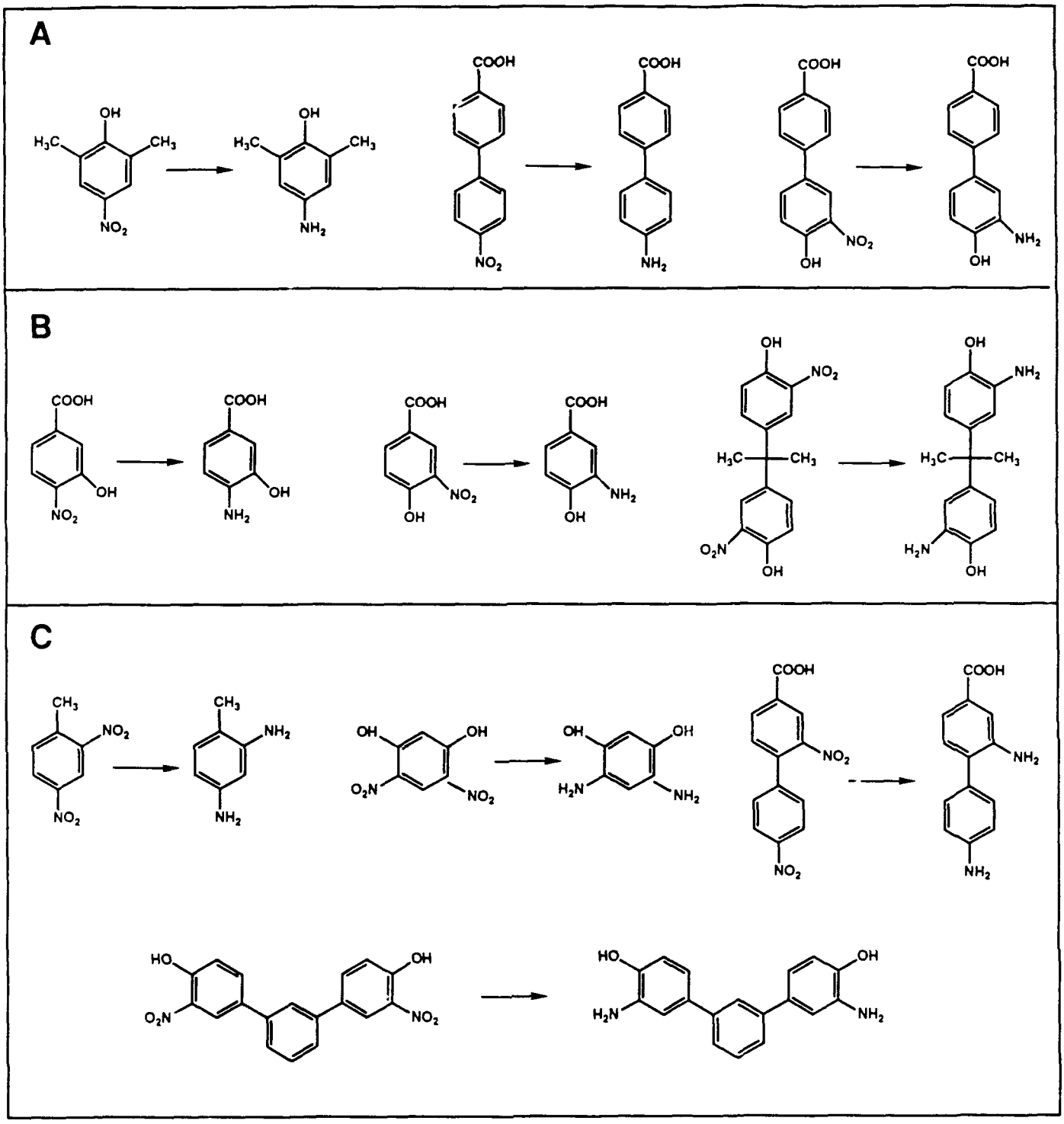


Figure 26. Substrates reduced by PI3A

(A.) Products have been acetylated, isolated, purified and examined by HPLC and NMR for verification of structure. (B.) Products have been identified based on HPLC data comparing the elution time and UV spectra of standards of known structure to the products from the reaction. (C.) Products are tentatively identified; data consists of HPLC retention times for products which are consistent with assigned structures.

The effect of pH on the rate of reduction was studied with the co-culture PI1/PI2. The reductions are done in minimal media containing 0.4% yeast extract, 450 ppm 4-hydroxy-3-nitrobenzoic acid, 20 ppm biphenyl-4-carboxylic acid (internal standard), and PI1/PI2 co-culture in a total volume of 150 ml. The pH varies from 6.9 to 8.6 in four separate flasks and is maintained by the addition of base metered into the flasks by a pump regulated by a pH controller. The reduction of substrate is quantified by HPLC analysis of aliquots of the reactions taken at various times. After the complete reduction of the initial 450 ppm of 4-hydroxy-3-nitrobenzoic acid, a second addition of 450 ppm of this substrate is added. The rate of reduction from this second addition is then monitored, and Figure 27 shows the effect of pH on the reduction rate. By determining the rate of reduction from the second addition of substrate after the cultures have reached their maximum growth, the rates can be compared from one flask to another. As shown in Figure 27, a pH from 7.6 to 7.8 appears to be optimum for the reduction of 4-hydroxy-3-nitrobenzoic acid to the corresponding 4-hydroxy-3-aminobenzoic acid. This is in contrast to the pH optimum of 8.5 to 9.0 for reduction of base-hydrolyzed 3-nitro-N-butylphthalimide, and implies that the pH optimum may be dependent on the nature of the substrate; this needs to be investigated further.

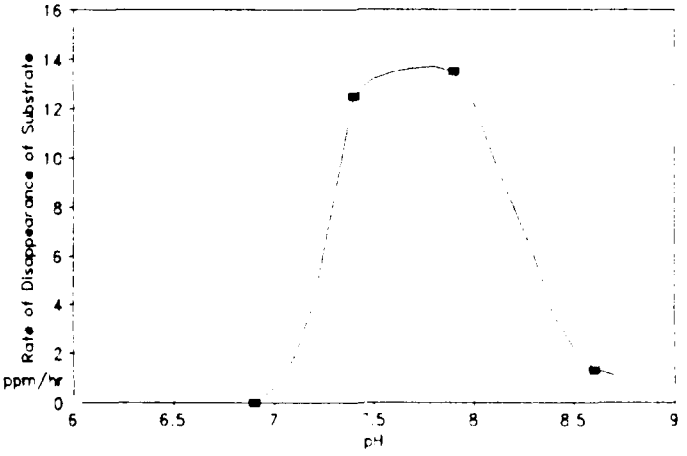


Figure 27. Effect of pH on the rate of reduction of 4-hydroxy-3-nitrobenzoic acid in a PI1 culture.

The standard conditions for nitroaromatic reduction with PI1 involved incubation of the substrate with the culture in minimal media with 0.4% yeast extract as the carbon source. Yeast extract is a complex mixture of compounds that would not be a practical substrate for large scale reactions because of its expense and because some of the components of yeast extract in the reaction mixture may interfere with product isolation and purification. In addition, an alternative carbon source may enhance the nitro-reductase activity. Several potential carbon sources were examined to determine the ability of PI1 (and PI2 in co-cultures) to utilize these compounds for growth and for nitro-reductase activity. These experiments were done in serum bottles filled 80% with minimal media at pH 8.5, 0.1% yeast extract, 500 ppm 4-hydroxy-3-nitrobenzoic acid, and one of the following compounds at 0.4% (wt:vol): succinate, glutathione, threonine, mannitol, fructose, or glucose. These were inoculated with either pure PI1 or a co-culture of PI1/PI2. The serum bottles were sealed, and the cultures grew aerobically until the oxygen in the bottles was depleted, at which point continued growth and reduction of 4-hydroxy-3-nitrobenzoic acid occurred under anaerobic conditions.

The optical densisty at 590 nm was monitored as a function of time with these cultures to determine extent of growth under these conditions (Figure 28). In conjuction, the nitro-reductase activity was determined with HPLC by observing the dissappearance of the substrate, 4-hydroxy-3-nitrobenzoic acid, and the formation of the corresponding reduced product (Table 12). Of the carbon sources monitored, mannitol provides the best combination of optimum growth and onset of nitro-reductase activity in both the PI1 and the co-culture of PI1/PI2. Glucose and fructose result in good growth in the co-culture, however with PI1 the growth is not as substantial. In all cases, glucose and fructose result in slower onset of nitro-reductase activity when compared with mannitol for a carbon source.

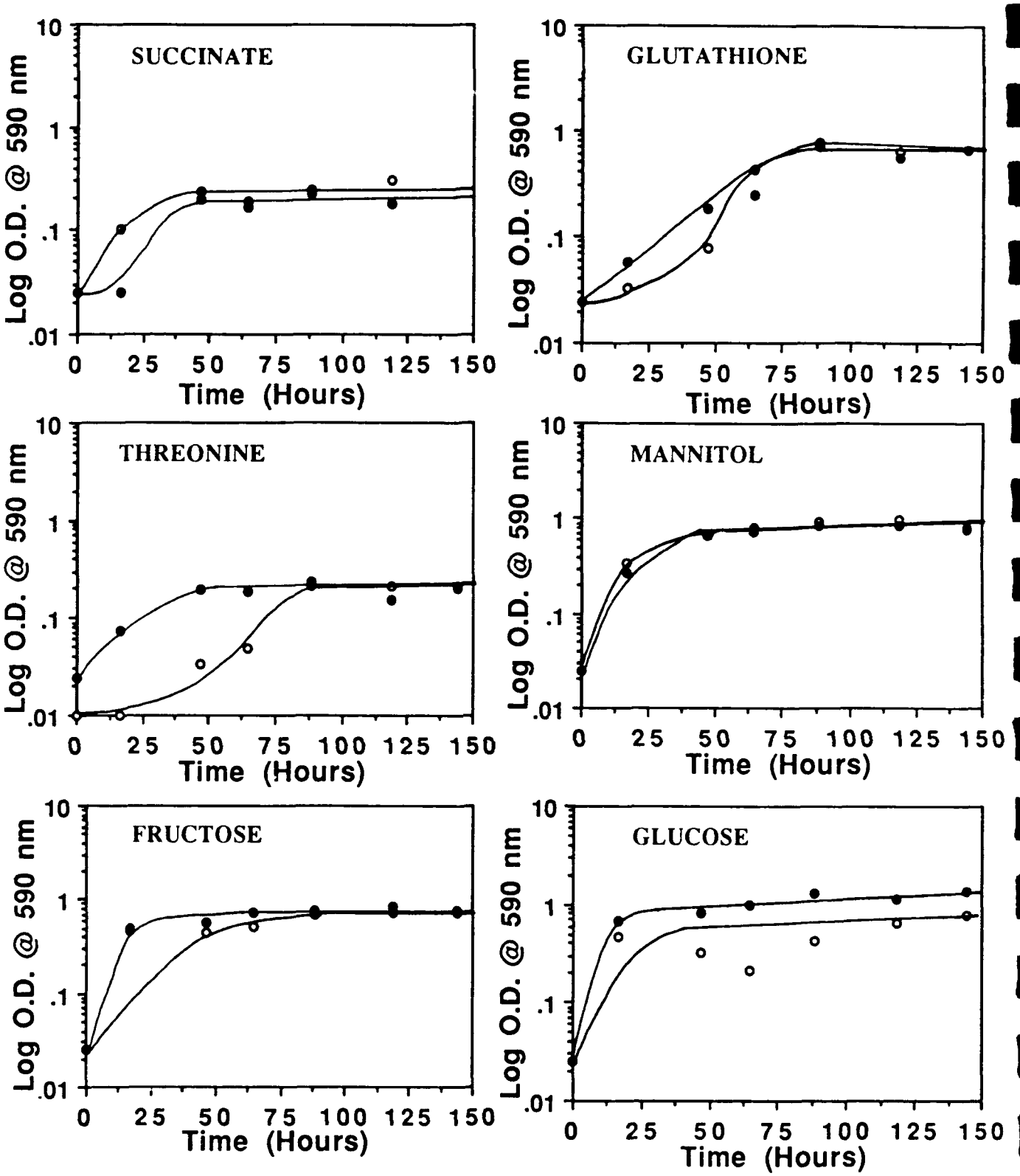


Figure 28. Anaerobic growth of strain PI1 and a co-culture of PI1/PI2 in a minimal media containing different carbon sources.

(●) indicates co-culture of PI1 and PI2.
(○) indicates pure culture of PI1.

Table 12. The effect of carbon source on the rate of reduction of 4OH-3NO_2-benzoic acid. (+) indicates that substrate is present. (0) indicates that substrate has been completely reduced.

	Time (Hours)					
Carbon Source	16.5	46.5	64.5	88.5	118.5	144
Succinate						
PI1	+	+	+	+	+	+
PI1+PI2	+	+	+	+	+	0
Glutathione						
PI1	+	+	+	+	+	+
PI1+PI2	+	+	+	+	+	+
Threonine						
PI1	+	+	+	+	+	+
PI1+PI2	+	+	+	+	+	+
Mannitol						
PI1	+	+	+	0	0	0
PI1+PI2	+	+	0	0	0	0
Fructose						
PI1	+	+	+	+	+	+
PI1+PI2	+	+	+	+	+	+
Glucose						
PI1	+	+	+	0	0	0
PI1+PI2	+	+	+	+	+	+
0.1% Yeast Extract						
PI1	+	+	+	+	+	+
PI1+PI2	+	+	+	+	+	+

5. CONCLUSION

The objectives for this work, as stated in the original research proposal, were: (1) to discover and evaluate biological routes to the syntheses of substituted o-aminophenols that would be advantageous over current chemical syntheses, and (2) to select the most favorable of these routes and develop a process for the large-scale biosynthesis of substituted o-aminophenols. To meet the first object, we proposed to study the ability of tyrosinase, p-hydroxybenzoate hydroxylase, salicylate hydroxylase, and p-aminobenzoate hydroxylase to catalyze the synthesis of o-aminophenol monomers. In addition to these isolated enzymes, we proposed to explore routes to the synthesis of o-aminophenols catalyzed by whole organisms.

The reactions catalyzed by tyrosinase, p-hydroxybenzoate hydroxylase, salicylate hydroxylase, and p-aminobenzoate hydroxylase and their mechanisms for catalysis suggested that these enzymes would catalyze the synthesis of o-aminophenol and, perhaps, substituted o-aminophenols. Indeed, we found that all of these enzymes can catalyze the synthesis of at least one o-aminophenol product. With tyrosinase, the substrate specificity is quite broad, and several different aminoaromatic compounds are ortho-hydroxylated. The products from tyrosinase-catalyzed reactions include o-aminophenol, 3,3'-dihydroxy-4,4'-diaminobiphenyl, and 4-amino-3-hydroxytoluene. With p-hydroxybenzoate hydroxylase, the only o-aminophenol synthesized is 4-amino-3-hydroxybenzoic acid. With salicylate hydroxylase, anthranilic acid is oxidatively decarboxylated to o-aminophenol. However, several other potential substrates appear only to promote NADH oxidation and do not yield o-aminophenol products. Finally, p-aminobenzoate hydroxylase catalyzes the synthesis of o-aminophenol from anthranilic acid, but does not produce substituted o-aminophneols when incubated with the appropriate substituted 2-hydroxybenzoic acid substrate.

Thus, the only two enzymes that catalyze the synthesis of potentially useful o-aminophenol monomers for polybenzoxazole production are tyrosinase and p-hydroxybenzoate hydroxylase. Of these two enzymes, p-hydroxybenzoate hydroxylase has the following two disadvantages: (1) NADPH, an expensive cofactor, is required for activity, and attempts to

overcome this issue by using whole organisms with the ability to provide both NADP and p-hydroxybenzoate hydroxylase were unsuccessful, and (2) substrate specificity is so narrow that only one o-aminophenol monomer, 4-amino-3-hydroxybenzoic acid, can be synthesized using this enzyme catalyst. For these reasons, most of the effort with isolated enzymes became focused on tyrosinase.

Tyrosinase has several other attractive features in addition to the broader substrate specificity: (1) it is relatively abundant and straightforward to isolate, (2) no expensive cofactors like NADPH or FAD need to be added to the reaction, (3) it is a relatively hardy enzyme, tolerating the presence of organic solvents in the reaction and, in some cases, catalyzing the hydroxylation in a water saturated organic solvent, and (4) it holds the promise of tolerating immobolization, thereby permitting certain reactor designs for continuous operation. The biggest disadvantage to be overcome is the ability of tyrosinase to catalyze the oxidation of o-aminophenols to iminoquinones, which are unstable and quickly react with substrate. We found that by choosing the appropriate source of tyrosinase, this oxidation can be overcome by the addition of ascorbic acid to the reaction. Thus, while mushroom tyrosinase is inhibited by the addition of ascorbic acid to the reaction containing an amino-aromatic compound, *Neurospora crassa* tyrosinase will catalyze the ortho-hydroxylation of amino-aromatic compounds in the presence of ascorbic acid to yield o-hydroxyamino-aromatic compounds. The addition of a reductant like ascorbic acid also helps stabilize the non-enzymatic oxidation of the desired o-aminophenols. Ascorbic acid, although convenient for preliminary studies, is probably not the most economical choice for large scale synthesis using tyrosinase; future work would have to survey alternative reductants that can be used in this tyrosinase-catalyzed hydroxylation of amino-aromatic compounds.

As an alternative to the use of isolated enzymes for the synthesis of o-aminophenol monomers, we investigated the ability to reduce o-nitrophenols using whole organisms. Biological reduction of o-nitrophenols using whole cells under anaerobic conditions offers advantages over chemical reductions, such as production of the oxidatively unstable o-aminophenol under aqueous, mild, oxygen-free conditions and simple product recovery due to the

neat conversion of the o-nitrophenol to the o-aminophenol. Using a GE bacterial strain PI1 and/or a co-culture of strain PI1 and PI2, we demonstrate that a broad range of nitroaromatic compounds can be reduced. Substrates that are reduced to the corresponding amino-aromatic compounds include 4-hydroxy-3-nitrobenzoic acid, 4,6-dinitroresorcinol, 4'-hydroxy-3'-nitrobiphenyl-4-carboxylic acid, and bis(4-hydroxy-3-nitrophenyl)propane. Nearly 100% conversions of the substrate to reduced product can be obtained, with final product concentrations as high as 8 g/L. Although this route to the synthesis of o-aminophenols looks promising, optimization of the culture conditions must be done to achieve greater reduction rates and higher product concentrations.

Our second objective, as stated in the original research proposal, was to select the most favorable of these routes and develop a process for the large-scale biosynthesis of substituted o-aminophenols. The most promising routes were ortho-hydroxylation of amino-aromatic compounds catalyzed by tyrosinase and reduction of o-nitrophenols catalyzed by bacterial strain PI1. Experiments were done to identify optimum conditions for both of these reactions. For example, the effect of ascorbic acid concentration and pH on the tyrosinase-catalyzed hydroxylations was examined, and the effect of product concentration, carbon source, pH, and temperature on the bioreduction of nitrophenols was examined. However, more work needs to be done with both of these catalysts before a large-scale synthetic approach is developed.

In conclusion, we have demonstrated that there are several routes to the biosynthesis of o-aminophenol. The most promising of these for the synthesis of o-aminophenol monomers are tyrosinase-catalyzed hydroxylation of amino-aromatic compounds and bioreduction of o-nitrophenols. The advantages of biosynthesis of o-aminophenol monomers by these routes include the production of the oxidatively unstable o-aminophenol under conditions that inhibit oxidation, the mild conditions (aqueous solution at low temperatures) required for the biocatalyzed reaction, the elimination of organic solvents in the syntheses, and the potentially simple product recovery process due to high conversions of substrate to product.

APPENDIX A

Physiology and Biochemistry: PI3AW and PI3AY

	PI3AW	PI3AY
Gram positive	+	+
Gram negative	—	—
Gram variable	—	—
Cells spherical	—	—
Cells coccobacillary	—	—
Cells in pockets	—	—
Cells in clusters	—	—
Cells in chains	+	+
Cells in tetrads	—	—
Endospores produced	—	—
Pigment diffusible	—	—
Pigment non-diffusible	—	—
Non-diffusible red	—	—
Non-diffusible yellow	—	—
A-haemolysis rabbit blood	—	—
B-haemolysis rabbit blood	—	—
A-haemolysis sheep blood	+	+
B-haemolysis sheep blood	—	—
Aerobic growth	+	+
Anaerobic growth	+	+
Microaerophilic	—	—
Increased CO_2 required	—	—
Serum required	—	—
Motile	—	—
10°C growth	+	+
37°C growth	+	+
45°C growth	+	+
3% NaCl growth	+	+
6.5% NaCl growth	+	+
10% NaCl growth	W	W
15% NaCl growth	—	—
10% bile growth	+	+
40% bile growth	+	+
pH 4.8 growth	—	+
pH 9.6 growth	+	+
0.1% methylene blue redn.	+	—
5 mcg optochin inhibition	—	
0.02% tellurite growth	+	+
0.02% lysostaphin lysis	—	—
Catalase	—	—
Oxidase	—	—
H_2O_2 produced	—	—
Coagulase	—	—
Indole	—	—
Methyl red	+	+
Vogues Proskauer	—	—
Simmons citrate growth	—	—

	PI3AW	PI3AY
Nitrate reduction	—	—
DL-arginine deamination	+	—
Hydrogen sulfide production	—	—
Phosphatase	+	—
Urease	—	—
Potassium cyanide growth	W	W
Aesculin hydrolysis	+	+
Casein hydrolysis	—	—
Gelatinase	—	—
Bacitracin inhibition	—	—
Starch hydrolysis	+	+
Tween 20 hydrolysis	—	—
Tween 80 hydrolysis	—	—
Tyrosine degradation	—	—
Hippurate hydrolysis	+	+
Deoxyribonuclease	—	—
Acid from:		
adonitol	—	+
maygdalin	+	+
L- arabinose	+	+
cellobiose	+	+
dulcitol	—	+
D- fructose	+	+
D- galactose	+	+
D- glucose AO_2	+	+
D- glucose AnO_2	+	+
glycerol	+	+
glycogen	—	+
i- inositol	—	+
inulin	+	+
lactose	+	+
maltose	+	+
D- mannitol AO_2	+	+
D- mannitol AnO_2	+	+
D- melezitose	+	—
melibiose	+	+
raffinose	+	+
L- rhamnose	+	+
D- ribose	+	+
salicin	+	+
D- sorbitol	+	+
sucrose	+	+
trehalose	+	+
D- xylose	+	+
Gas from: D-glucose	—	—

W = weakly positive

APPENDIX B

Biolog GN Microplate Activity

A1	Water	—	D1	Acetic acid	+
A2	α-cyclodexrin	—	D2	Cis aconitic acid	+
A3	Dextrin	—	D3	Citric acid	+
A4	Glycogen	—	D4	Formic acid	+
A5	Tween 40	+	D5	D-galactonic acid lactone	—
A6	Tween 80	+	D6	D-galacuronic acid	—
A7	N-acetyl-D-galactosamine	—	D7	D-Gluconic acid	—
A8	N-acetyl-D-glucosamine	—	D8	D-glucosaminic acid	—
A9	Adonitol	—	D9	D-glucuronic acid	—
A10	L-arabinose	—	D10	α-hydroxybutyric acid	+
A11	D-arabitol	—	D11	β-hydroxybutyric acid	+
A12	Cellobiose	—	D12	γ-hydroxybutyric acid	+
B1	i-erythritol	—	E1	p-hydroxyphacylacetic acid	+
B2	D-fructose	—	E2	Itaconic acid	—
B3	L-frucose	—	E3	α-keto butyric acid	+
B4	D-galaciose	—	E4	α-keto glutaric acid	—
B5	Genatiobiose	—	E5	α-keto valeric acid	—
B6	α-D-glucose	—	E6	D,L-lactic acid	—
B7	m-inositol	—	E7	Malonic acid	—
B8	α-lactose	—	E8	Propionic acid	—
B9	Lactulose	—	E9	Quinic acid	—
B10	Maltose	—	E10	D-saccharic acid	—
B11	D-mannitol	—	E11	Sebacic acid	—
B12	D-mannose	—	E12	Succinic acid	+
C1	D-melibiose	—	F1	Bromo succinic acid	+
C2	β-methyl glucoside	—	F2	Succinamic acid	+
C3	Psicose	—	F3	Glucuronamide	—
C4	D-raffinose	—	F4	Alaninamide	—
C5	L-rhamnose	—	F5	D-alanine	—
C6	D-sorbitol	—	F6	L-alanine	—
C7	Sucrose	—	F7	L-alanylglycine	—
C8	D-trehalose	—	F8	L-asparagine	+
C9	Turanose	—	F9	L-aspartic acid	+
C10	xylitol	—	F10	L-glutamic acid	+
C11	methyl pyruvate	+	F11	Glycly-L-aspartic acid	—
C12	Mono-methyl succinate	+	F12	Glycly-L-glutamic acid	—

G1	L-histidine	+
G2	Hydroxy-L-proline	—
G3	L-leucine	+
G4	L-ornithine	+
G5	L-phenylalanine	+
G6	L-proline	+
G7	L-pyroglutamic acid	+
G8	D-serine	+
G9	L-serine	+
G10	L-threonine	+
G11	D,L-carnitine	—
G12	γ-aminobutyric acid	—
H1	Urocanic acid	—
H2	Inosine	+
H3	Uridine	—
H4	Thymidine	—
H5	Phenylethylamine	+
H6	Putrescine	—
H7	2-amino ethanol	—
H8	2,3-butanediol	—
H9	Glycerol	—
H10	D,L-α-glycerol phosphate	—
H11	Glucose-1-phosphate	—
H12	Glucose-6-phosphate	—

REFERENCES

1. U.S. Patent 4,533,692 (August 6, 1985), J. F. Wolfe, P. D. Sybert and J. R. Sybert (to SRI International).

2. U.S. Patent 4,533,693 (August 6, 1985), J. F. Wolfe, P. D. Sybert, and J. R. Sybert (to SRI International).

3. U.S. Patent 4,533,724 (August 6, 1985), J. F. Wolfe, P. D. Sybert, and B. Wilson (to SRI International).

4. Wolfe, J. F., in *Encyclopedia of Polymer Science and Engineering*, Vol. 11, pp 601-635, John Wiley & Sons (1988).

5. Moyer, W. W., Jr., Cole, C. and Anyos, T., *J. Polymer Sci. A3*, 2107-2121 (1965).

6. Imai, Y., Toako, I., Uno, K. and Iwakura, Y., *Makromol. Chem. 83*, 167-178 (1965).

7. Beck, S. D. and Smissman, E. E., *Ann. Entomol. Soc. Am. 54*, 53-61 (1961).

8. Odle, R., Barren, J. and Shanklin, E. W., personal communication and internal GE documents.

9. Theriault, R. J. and Longfield, T. H., *Appl. Microbiol. 15*, 1431-1436 (1967).

10. Smith, R. V., *Biotech. Bioeng. 17*, 785-814 (1975).

11. Smith, R. V. and Rosazza, J. P., *Arch. Biochem. Biophys. 161*, 551-558 (1974).

12. Hallas, L. E. and Alexander, M., *Appl. Environ. Microbiol. 45*, 1234-1241 (1983).

13. Naumova, R. P., Amerkhanova, N. N. and Belousoua, T. O., *Mikrobiologiya 51*, 725-739 (1982).

14. Naumova, R. P., Amerkhanova, N. N. and Zolotukhina, L. M., *Prikladnaya Biokhimiya i Mikrobiologiya 19*, 507-512 (1983).

15. Kinouchi, T. and Ohnishi, Y., *Appl. Environ. Microbiol. 46*, 596-604 (1983).

16. McCormick, J. R. D., Reichenthal, J., Hirsch, U. and Sjolander, N. O., *J. Am. Chem. Soc. 84*, 3711-3714 (1962).

17. Vanneste, W. H. and Zuberbuhler, A., In *Molecular Mechanisms of Oxygen Activation*; Hayaishi, O., Ed.; Academic Press, New York 1974; pp 371-404.

18. Robb, D. A., In *Copper Proteins and Copper Enzymes*, Lontie, R., Ed., CRC Press, Boca Raton, FL, 1984; Vol. ?, pp 207-241.

19. Engelsma, G. and Havinga, E., *Tetrahedron 2*, 289-295 (1958).

20. Mason, H.S., *Adv. Enzymol. 16*, 105 (1955).

21. Mayer, A. M. and Harel, E., *Phytochemistry 18*, 193 (1979).

22. Bouchilloux, S., McMahill, P. and Mason, H. S., *J. Biol. Chem. 238*, 1699-1707 (1963).

23. Pomerantz, S. H. and Murthy, V. V., *Arch. Biochem. Biophys. 160*, 73-82 (1974)..

24. Miller, L. O., Newcombe, R. and Triplett, E. L., *Comp. Biochem. Physiol. 32*, 555-567 (1970).

25. Balasingam, K. and Ferdinand, W., *Biochemical J. 118*, 15-23 (1970).

26. Lerch, K., *Methods in Enzymology*; Academic Press, New York, 1987; Vol. 142, pp 165-169.

27. Robb, D. A., *Biochem. Soc. Trans. 7*, 131-132 (1979).

28. Toussaint, O. and Lerch, K., *Biochemistry 26*, 8567-8571 (1987).

29. Vilanova, E., Manjon, A. and Iborra, J. L., *Biotech. Bioeng. 26*, 1306-1312 (1984).

30. Patil, S. S. and Zucker, M., *J. Biol. Chem. 240*, 3938-3943 (1965).

31. Lerch, K., private communication.

32. Wilcox, D. E., Porras, A. G., Hwang, Y. T., Lerch, K., Winkler, M. and Soloman, E. I., *J. Am. Chem. Soc. 107*, 4015-4016 (1985).

33. Howell, L. G., Spector, T., and Massey, V., *J. Biol. Chem. 247*, 4340-4350 (1972).

34. Hosokawa, K. and Stanier, R. Y., *J. Biol. Chem. 241*, 2453-2460 (1966).

35. Yano, K., Higashi, N. and Arima, K., *Biochem. Biophys. Res. Commun. 34*, 1-7 (1969).

36. Michalover, J. L. and Ribbons, D. W., *Biochem. Biophys. Res. Commun. 55*, 888-896 (1973).

37. Fujii, T. and Kaneda, T., *Eur. J. Biochem. 147*, 97-104 (1985).

38. Entsch, B., Ballou, D. P. and Massey, V., *J. Biol. Chem. 251*, 2550-2563 (1976).

39. Ghiala, S., Entsch, B., Massey, V. and Hussain, M., *Eur. J. Biochem. 76*, 139-148 (1977).

40. Claiborne, A. and Massey, V., *J. Biol. Chem. 258*, 4919-4925 (1983).

41. Shoun, H. and Beppu, T., *J. Biol. Chem. 257*, 3422-3428 (1982).

Entsch, B., Hussain, M., Ballou, D.P., Massey, V. and Walsch. C., *J. Biol. Chem. 255*, 1420-1429 (1980).

43. Hussain, M. and Massey, V., *J. Biol. Chem. 254*, 6657-6666 (1979).

44. Schreuder, H. A., van der Laan, J. M., Hol, W. G. J. and Drenth, J., *J. Mol. Biol. 199*, 637-648 (1988).

45. Entsch, B., Nan, Y., Weaich, K. and Scott, K. F., *Gene 71*, 279-291 (1988).

46. Yamamoto, S., Katagiri, M., Maeno, H., and Hayaishi, O., *J. Boil. Chem. 240*, 3408-3413 (1965).

47. Takemori, S., Yasuda, H., Mihara, K., Suzuki, K., and Katagiri, M., *Biochim. Biophys. Acta 191*, 58-68 (1969).

48. White-Stevens, R. H. and Kamin, H., *J. Biol. Chem. 247*, 2358-2370 (1972).

49. Szem I. S.-Y., and Dagley, S., *J. Bacteriol. 159*, 353-359 (1984).

50. Katagiri, M., Takemori, S., Suzuki, K., and Yasuda, H., *J. Biol. Chem. 241*, 5675-5677 (1966).

51. White-Stevens, R. H. and Kamin, H., *J. Biol. Chem. 247*, 2371-2381 (1972).

52. Tsuji, H., Ogawa, T., Bando, N. and Sassaoke, K., *Biochem. Biophys. Res. Commun. 130*, 633-639 (1985).

53. Tsuji, H., Ogawa, T., Bando, N. and Sassaoke, K., *J. Biol. Chem. 261*, 13203-13209 (1986).

54. McCormick, N. G., Feeherry, F. E., and Levinson, H. S., *Appl. Environ. Microbiol. 31*, 949-958 (1976).

55. Villanueva, J. R. *J. Biol. Chem. 254*, 6657-6666 (1979).

56. Zucker, M. and Nason, A., *Methods in Enzymology*; Academic Press: New York 1955; Vol. 2, pp 416-419.

57. Higgins, E. S., *Fed. Proc. 17*, 242-244 (1958).